JULIANA DE OLIVEIRA

POLY(LACTIC ACID) PRODUCTION BY CONVENTIONAL AND MICROWAVE POLYMERIZATION OF LACTIC ACID PRODUCED IN SUBMERGED FERMENTATION

Thesis submitted in partial fulfillment of the requirements for the degree of doctor in Bioprocess Engineering and Biotechnology, Agroindustry Area. Graduation Program in Bioprocess Engineering and Biotechnology, Federal University of Parana.

Supervisor: Luciana Porto de Souza Vandenberghe, Ph.D.

Co-Supervisors: Carlos Ricardo Soccol, Ph.D.

Sônia Faria Zawadzki, Ph.D.

CURITIBA

2016

UNIVERSIDADE FEDERAL DO PARANA
Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia
Setor de Tecnologia

UFPR

RELATÓRIO DE DEFESA DE TESE DE DOUTORADO

Aos nove dias do mês de Junho de 2016, na Sala 3 – Prédio da Engª de Bioprocessos e Biotecnologia, CENBAPAR, do Centro Politécnico da Universidade Federal do Paraná, Jardim das Américas, foi instalada pelo Prof. Dr. Júlio Cesar de Carvalho, Coordenador do programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, a banca examinadora para a Centésima Décima Terceira Defesa de Tese de Doutorado. Área de Concentração: Agroindústria e Biocombustíveis. Estiveram presentes no Ato, além do coordenador do programa, professores, alunos e visitantes.

A Banca Examinadora, atendendo determinação do Colegiado do Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, ficou constituída pelos Professores Doutores Luciana Porto de Souza Vandenberghe – orientadora da tese, UFPR, Carlos Ricardo Soccol – co-orientador da tese – UFPR, Sônia Faria Zawadzki- co-orientadora da tese - UFPR, Satinder Kaur Brar – INRS, Canadá, Júlio César de Carvalho – UFPR e Valcineide Tanobe - UFPR.

Às 09h, a banca iniciou os trabalhos, convidando a candidata **Juliana de Oliveira,** a fazer a apresentação da Tese intitulada: "POLY(LACTIC ACID) PRODUCTION BY CONVENTIONAL AND MICROWAVE POLYMERIZATION OF LACTIC ACID PRODUCED IN SUBMERGED FERMENTATION OF BRAZILIAN ALTERNATIVE SUB-PRODUCTS". Encerrada a apresentação, iniciou-se a fase de arguição pelos membros participantes.

Tendo em vista a tese e a arguição, a banca composta pelos professores doutores. Luciana Porto de Souza Vandenberghe, Carlos Ricardo Soccol, Satinder Kaur Brar, Sônia Faria Zawadzki, Júlio César de Carvalho e Valcineide Oliveira de Andrade Tanobe, declarou a candidata APROVADA (de acordo com a determinação dos Artigos 59 a 68 da Resolução 65/09 de 30.10.09).

Curitiba, 09 de Junho de 2016.

Profª. Dra. Luciana P. S. Vandenberghe

Prof. Dr. Carlos Ricardo Soccol

Profª Dra. Satinder Kaur Brar

Profa. Dra Sônia Faria Zawadzki

Profa. Dra. Valcineice O. A. Tanobe

Prof. Dr. Júlio César de Carvalho

ACKNOWLEDGEMENT

I must offer my gratitude to my supervisors Dr. Luciana Porto de Souza Vandenberghe and Dr. Carlos Ricardo Soccol for providing necessary infrastructure and resources to realize my research work and supervision, help and guidance during development of my thesis. I would like to thank Dr. Sonia Faria Zawadzki for co-supervision and infrastructure for polymerization studies.

I thank Dr. Satinder Kaur Brar by reception on her team, supervision on work done at the INRS and also Dr. Tarek Rouissi for his collaboration.

I must thank Dr. Valcineide Tanobe for help in analyzes and advice, constructive criticism and discussions on my work.

The interns Karina Kawka and Rafael Lobo for help in carrying out the work.

Friends and colleagues Mitiyo, Otacílio, Mario, Nelson, Patrick, Dao, Adriano for support at all times. Co-workers, Biotechnological Processes Laboratory of teachers.

CAPES for financial support and available material resources. The UFPR and the Graduate Program in Bioprocess Engineering Biotechnology by the structure necessary to carry out this work.

My family for emotional support, the prayers and all the support.

ABSTRACT

Poly (lactic acid) (PLA) is a polyester, which has a predominant role as biodegradable plastic, that is applied in packaging, textile, medical and pharmaceutical products. It can be obtained from lactic acid by direct polycondensation and by ring-opening polymerization (ROP) of lactide. Lactic acid (LA) is an organic acid that presents diverse applications mostly in food industry, as well as in pharmaceutical, chemical industries and polymers. The production of LA by fermentation offers the advantage of producing optically high pure LA. Nutritional requirements of bacteria increase the cost of LA production so alternatives substrates have been studied to bring an economical alternative for this process. The aim of this work was the production of LA by *Lactobacillus pentosus* in submerged fermentation using potato processing waste and sugarcane juice as substrate in order to obtain poly(lactic acid). The fermentation process was developed using potato processing waste and sugarcane juice because of their high carbon source concentration. Important volumes of both sub-products were generated, which is another reason for their re-use and valorization. Potato processing waste was submitted to hydrolysis in order to convert starch to glucose. LA production by fermentation was optimized using, statistical experimental design approach steps of optimization involved the screening of bacteria of the genus *Lactobacillus* and definition of medium composition kinetics studies in Erlenmeyer flask and stirred tank reactor were also carried out. LA production reached 150 g/l using potato processing waste, it was and 225 g/l with sugar cane juice after 96 hours of fermentation. The use of baker's yeast as a source of nitrogen and nonsterile conditions demonstrated good alternatives for an industrial production process of LA. The separation and recovery process of LA from fermented broth was developed to obtain a purified molecule for further polymerization studies. The developed process consisted in heating the fermented broth, then a centrifugation step was conducted for removal of the cells and suspended solids. A clarification step was included with powered activated carbon with further precipitation at low temperature and acidification of calcium lactate to convert to LA. The process was effective for removal of contaminants that were present in the fermentation medium. Final concentration of LA in aqueous solution reached 416 g/l and a yield of 51%. Polymerization studies were then carried out using direct polycondensation of LA, that were carried out with two different heating systems, conventional and microwave heating. A polymer with 6330 g/mol of molecular weight and 61% of yield was obtained from commercial LA and using fermented LA resulted in 2370 g/mol. Microwave heating process provided a higher yield, 79% and 76% for commercial and fermented LA, respectively. Nevertheless, the molecular weight was lower than conventional process, 2070 for commercial LA and 1450 for fermented LA. Physicochemical properties of PLA demonstrated application in encapsulation of bioactive compounds and tissue engineering. Perspectives of sequence of the studies: application on encapsulation of molecules, modifications of polymer and development of composites.

KEYWORDS: Poly(lactic acid); potato processing waste; sugarcane juice; polycondensation

RESUMO

Poli(ácido lático), poliéster, é um polímero biodegravável aplicado em produtos como embalagens, têxteis, médicos e farmacêuticos. Pode ser obtido a partir do monômero ácido lático (AL) por meio da reação de policondensação direta e pela polimerização por abertura de anel do lactídeo. O AL é um ácido orgânico que apresenta diversas aplicações principalmente na indústria alimentícia, assim como na indústria farmacêutica, química e de polímeros. A produção do AL por fermentação oferece vantagens tais como a produção do isômero opticamente puro. As necessidades nutricionais da bactéria aumentam o custo de produção do AL, portanto substratos alternativos tem sido estudados por apresentarem uma alternativa econômica para este processo. O objetivo deste trabalho foi a produção de ácido lático por *Lactobacillus pentosus* em fermentação submersa utilizando subproduto do processamento da batata e caldo de cana como substratos para a obtenção de poli(ácido lático). Estes sub-produtos porque possuem alta concentração de fonte de carbono e volumes significativos são gerados anualmente, o que justifica sua a re-utilização e valorização. O sub-produto do processamento da batata foi submetido a hidrólise ácida com o objetivo de converter o amido em glucose. A produção de AL foi otimizada utilizando etapas de planejamento experimental estatístico envolvendo a seleção de bactérias do gênero *Lactobacillus*, definição da composição do meio de cultivo e estudos de cinética em frascos de Erlenmeyer e biorreator do tipo tanque agitado. A produção de AL chegou a 150 g/L utilizando sub-produto do processamento da batata e 225 g/L utilizando caldo de cana em 96 horas de fermentação. O uso da célula inteira de levedura de panificação como fonte de nitrogênio e a condição de fermentação não estéril demostraram ser boas alternativas para um processo industrial de produção de AL. O processo de separação e recuperação do AL do caldo fermentado foi desenvolvido para obtenção da molécula purificada e estudos de polimerização com o monômero obtido. O processo desenvolvido consistiu no aquecimento do caldo fermentado seguido pela etapa de centrifugação. A etapa de clarificação foi realizada utilizando carvão ativado em pó seguida pela precipitação a baixa temperatura e acidificação do lactato de cálcio para conversão em ácido lático. O processo foi efetivo para remoção de contaminantes que estavam presentes no caldo fermentado. A concentração final de AL em solução aquosa foi de 416 g/L com um rendimento de 51%. Os estudos de polimerização foram desenvolvidos utilizando a técnica de policondensação direta do AL, por meio de dois diferentes sistemas de aquecimento, convencional e micro-ondas. Um polímero com massa molar de 6330 g/mol e 61% de rendimento foi obtido a partir de um AL comercial e utilizando o AL obtido por fermentação resultou em um polímero com massa molar de 2370 g/mol. O processo de aquecimento por micro-ondas proporcionou um maior rendimento, 79% e 76% para o AL comercial e obtido por fermentação, respectivamente. Porém, foi obtida menor massa molar que o processo convencional, 2070 para o AL comercial e 1450 para o AL obtido por fermentação. As propriedades físico-químicas do poli(ácido lático) demonstraram aplicação em encapsulamento de compostos bioativos e engenharia de tecido. As perspectivas de sequência de estudos são a aplicação em encapsulamento de moléculas, modificações do polímeros e desenvolvimento de compósitos.

PALAVRAS CHAVE: Poli(ácido lático), sub-produto do processamento da batata, caldo de cana, policondensação

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BYC	baker's yeast cell
C/N	carbon/nitrogen ratio
DSC	Differential scanning calorimetry
FTIR	Fourier Transform Infrared
GPC	Gel permeation chromatography
HPLC	High performance liquid chromatography
HPPW	Hydrolyzed potato processing waste
LA	Lactic acid
LAB	Lactic acid bacteria
MRS	Man, Rogosa and Sharpe medium
Mv	Viscosimetric molecular weight
MW	Microwave
PAC	powdered activated carbon
PLA	Poly(lactic acid)
PLACC	Poly(lactic acid) from commercial monomer and conventional polymerization
PLACMW	Poly(lactic acid) from commercial monomer and microwave polymerization
PLAFC	Poly(lactic acid) from fermented monomer and conventional polymerization
PLAFMW	Poly(lactic acid) from fermented monomer and microwave polymerization
PPW	Potato processing waste
ROP	Ring opening polymerization
SCJ	Sugar cane juice
SEM	Scanning electron microscopy
STR	Stirred tank reactor
Tg	Glass transition temperature
TGA	Thermogravimetric analysis
Tm	Melting temperature
XRD	X-ray diffraction
YE	Yeast extract

LIST OF FIGURES

LIST OF TABLES

CONTENTS

INTRODUCTION

Polymers from fossil fuels generates significant amounts of waste, which accumulate in the ecosystem resulting in a serious environmental problem. In general, synthetic polymers are resistant to chemical and/or physical degradation, as well as they present resistance to microbial degradation (Fang *et al.*, 2005; Leja and Lewandowicz, 2010). Polymers produced from natural resources become attractive to reduce the consumption of petroleum based non-renewable polymers.

Biodegradable polymers can be degraded by organisms such as bacteria, fungi and algae (Kumar *et al.*, 2010), however the biodegradability of polymers depends on the molecular weight, molecular form and crystallinity (Premraj and Doble, 2005). There is a wide variety of biodegradable polymers in development, among them the polyesters such as polyhydroxyalkanoates (PHA), polyhydroxyhexanoate (PHH), polyhydroxybutyrate (PHB), polyhydroxyvalerate (PHV), polylactic acid (PLA), polycaprolactone (PCL), polybutylene (PBS) succinate, polybutylene succinate adipate (PBSA), aliphatic–aromatic copolyesters (AAC), polybutylene adipate/terephthalate (PBAT) and polymethylene adipate/terephthalate (PTMAT) (Madhavan Nampoothiri *et al.*, 2010).

Polymerization of lactic acid produces poly(lactic acid) (PLA), a polymer that can be applied in the areas of packaging, textile, biomedical and pharmaceutical. PLA has properties comparable to existing petroleum-based polymers such as high strength, modulus and transparency, etc. (Lim *et al.*, 2008; Liu *et al.*, 2011). However, properties of PLA such as the thermal properties, toughness, water vapor and gas barrier are inferior to those of conventional petroleum-based polymers. Combining the polymer with reinforcing elements, thus making blends and composites, is a strategy to improve the properties of PLA (Fortunati *et al.*, 2012).

Commercial production of PLA has been done by ring opening polymerization of lactide, which high molecular weight is reached. This method involves high costs due to the production and purification process of the lactide (Madhavan Nampoothiri *et al.*, 2010). Direct polycondensation of lactic acid results in low molecular weight due to the difficulty on removal of water formed during the reaction. It is essential to ensure the water withdrawal to obtain high molecular weight polymers because the presence of water can enhance a hydrolysis reaction of the polymer formed (Gupta and Kumar, 2007; Marques *et al.*, 2010; Södergärd and Stolt, 2010).

Lactic acid (CH_3-CHOHCOOH) is an organic acid discovered in 1780 by Swedish chemist Scheele from sour milk as a brown syrup and named ’Mjölksyra’. In 1789, Lavoisier

gave the name “acide lactique”. Only in 1857, Pasteur discovered that lactic acid was a fermentation product of some microorganisms (Ghaffar *et al.*, 2014).

Lactic acid is used in food, pharmaceutical, cosmetic, chemical and others industries for several decades. Due to its versatile applications as flavoring, inhibitor of bacterial, acidulant, lactic acid is considered one of the most important hydroxycarboxylic acids. Besides, lactic acid presents carboxylic and hydroxyl groups in its structure, it can also be converted into different potentially useful chemicals such as pyruvic acid, acrylic acid, esters, solvents, among others (Datta and Henry, 2006; Gao *et al.*, 2011).

The molecule has an asymmetric carbon, it exists in the form of two optically active isomers: the L(+)lactic acid and D(-)lactic acid. Chemical synthesis produces racemic mixture of DL-lactic acid. In this case, the synthesis of lactic acid is mainly based on the hydrolysis of lactonitrile, a derivative of petrochemicals. An optically pure L (+) - or D (-) - lactic acid is obtained by fermentation (Narayanan *et al.*, 2004). Bacteria and fungi have been reported as microorganisms that produce LA. The LA bacteria consists of a group of bacteria, which produces LA as a major metabolic product (Reddy *et al.*, 2008).

Carbon sources used for LA production can be sugars such as glucose, sucrose, lactose, etc. These carbon sources have a higher cost, so the use of substrates containing carbon sources such as cassava bagasse, sugar cane bagasse, molasses, whey, starches, among others can reduce the cost of LA production (Madhavan Nampoothiri *et al.*, 2010).

The production of biopolymers from renewable sources is certainly an important alternative to reduce human dependence on fossil fuels. Moreover, lactic acid is a raw material for the production of polymers. Based on this premise, this work aims Production of lactic acid by fermentation by *Lactobacillus pentosus* using potato waste processing and sugarcane juice the substrate in order to obtain poly(lactic acid).

OBJECTIVES

Main objective

Production of lactic acid in submerged fermentation by *Lactobacillus pentosus* using potato processing waste and sugarcane juice as substrate in order to obtain poly(lactic acid) through two different polymerization methods.

Specifics objectives

- Screening of *Lactobacillus* strains for LA production.
- Development of lactic acid process production using two alternative sub-products, potato processing waste and sugarcane juice.
- Recovery and purification of LA.
- Development of a polymerization process by conventional heating process.
- Development of a polymerization process by microwave heating process.
- Characterization of poly(lactic acid) polymers obtained from different polymerization methodologies through advanced analytical methods (Differential scanning calorimetry (DSC), Fourier Transform Infrared (FTIR), X-ray Diffraction (XRD), Thermogravimetric Analysis (TGA) and Scanning Electron Microscopy (SEM)).

CHAPTER 1 – LITERATURE REVIEW

1.1 LACTIC ACID

1.1.1 Production

Lactic acid can be produced by chemical synthesis or biotechnological processes. The lactic acid produced by chemical synthesis is a racemic mixture of the isomers L(-)- and D(+)-lactic acid and this process is mainly based on the hydrolysis of lactonitrile with the use of strong acids. Other chemical routes, such as base-catalyzed degradation of sugars; oxidation of propylene glycol; reaction of acetaldehyde, carbon monoxide, and water at high temperatures and pressures; hydrolysis of chloropropionic acid; and nitric acid oxidation of propylene, are not technically and economically feasible processes for lactic acid production (Gao *et al.*, 2011).

Biotechnological processes offer advantages as optically high pure lactic acid by selecting an appropriate strain. Pure isomers, L(-)- and D(+)-lactic acid, are more valuable than the racemic form because each isomer has its own specific industrial application (Abdel-Rahman *et al.*, 2011). Furthermore, biotechnological processes use alternative raw materials, considered as attractive alternative substrates and renewable resources, including byproducts of agricultural industries, food industries, and natural unutilized biomasses such as starchy biomass, lignocellulosic biomass, whey, yogurt, glycerol, and algal biomass (Abdel-Rahman *et al.*, 2013).

Presently, almost all lactic acid produced globally is manufactured by fermentation process (Abdel-Rahman *et al.*, 2011). The global lactic acid production in 2013 was 714.2 kilo tons and it is expected 1,960.1 kilo tons in 2020, at a CAGR (compound annual growth rate) of 15.5% from 2014 to 2020. Global lactic acid market reached USD 1,285.6 million in 2013 and it is expected to reach USD 4,312.2 million by 2020 (Research, 2015).

Substrates as glucose, sucrose and lactose are carbon sources mostly used for lactic acid fermentation, nevertheless the cost of these carbohydrates becomes the fermentation process economically unfavorable. Materials such as molasses have been exploited as cheaper alternatives due to low cost (John *et al.*, 2007; Lunelli *et al.*, 2009). Other materials as starch and lignocelluloses, from agricultural residues and forestry resources, are cheap and show the

requirements for the biotechnological production of lactic acid (Abdel-Rahman *et al.*, 2011; Budhavaram and Fan, 2009; Talukder *et al.*, 2012). Lignocellulosic hydrolysates materials have also been reported for LA production, however a major issue is to develop organisms that utilize the available sugars including hexoses and pentoses (Hofvendahl and Hahn–Hägerdal, 2000; Tanaka *et al.*, 2002). Some developed processes for lactic acid production by fermentation are shown in Table 1-1.

Table 1-1 Biotechnological process for lactic acid production using different substrates and microorganisms

Microorganism	Substrate	LA Production	Reference
Enterococcus mundtii QU 25	Glucose/xylose	129.0 g/l	(Abdel-Rahman *et al.*, 2015)
Lactobacillus plantarum mutant	Cellulosic feedstocks	55.2 – 84.6 g/l	Hama et al. (2015)
Rhizopus oryzae	Waste residue from corncob after xylo-oligosacharides manufacturing	34.0 g/l	Zhang et al (2015)
Bacillus sp. WL-S20	Peanut meal and glucose	225.0 g/l	(Meng *et al.*, 2012)
Lactobacillus paracasei	Glucose	192.0 g/l	(Moon *et al.*, 2012)
Escherichia coli strain CICIM B0013-070	Glicerol	111.5 g/l	(Tian *et al.*, 2012)
Lactobacillus agilis	Soybean vinasse	138.0 g/l	(Karp *et al.*, 2011)
Sporolactobacillus sp. strain CASD	Peanut meal and glucose	207.0 g/l	(Wang *et al.*, 2011)
Rhizopus oryzae GY18	Glucose	115.0 g/l	(Guo *et al.*, 2010)
Bacillus sp Na-2	Glucose	106.0 g/l	(Qin *et al.*, 2010)
Lactobacillus rhamnosus	Cassava powder	175.0 g/l	(Wang *et al.*, 2010)

A limiting factor for efficiency of lactic acid fermentation processes is related to use of waste because the most renewable materials need pretreatment due to their intimate association with lignin and the lack of hydrolytic enzyme production by lactic acid-producing strains (Abdel-Rahman *et al.*, 2011). The conversion of starch or cellulose to sugar consumes energy during saccharification and increases the production costs. Another limiting factor is the recovery and purification of lactic acid from the fermentation broth, because complex media hampers the separation and purification of the acid.

In this sense, different studies have been conducted using different operational modes such as batch, fed-batch, semi-continuous/repeated batch, and continuous fermentation. Higher concentration of lactic acid was achieved in batch and fed-batch cultures than in others, whereas higher productivity was obtained in continuous cultures. Although batch fermentation is the most used for lactic acid production, problems as low productivity due to long fermentation times and low cell concentrations, and end product inhibition are observed. Comparatively,

batch fermentation exhibited higher lactic acid concentration and yield than continuous fermentation, but lower lactic acid productivity. Changes in fermentation systems can significantly improve the results. For example, by integrating a cell recycling system with continuous fermentation, higher cell density can be achieved, and this strategy drastically increases lactic acid productivity (Tashiro *et al.,* 2011). One advantage of the continuous processes over batch cultures is that the process can be run for longer periods of time (Vijayakumar *et al.*, 2008).

1.1.2 Microorganisms

Lactic acid can be produced by several microorganisms, including bacteria, fungi, yeast, algae, and cyanobacteria. The selection of the strain is of great importance, particularly in terms of their capacity in secreting high optical purity of lactic acid, reduced nutritional requirements and promoting high yields and productivities. Lactic acid fermentation processes to lactic acid can be classified according to the type of lactic acid bacteria (LAB) that is used. They can be homofermentative or heterofermentative. In the heterofermentative process, equimolar amounts of lactic acid, acetic acid, ethanol, and carbon dioxide are formed from hexose, whereas in the homofermentative process only lactic acid is produced from hexose metabolism (Hofvendahl and Hahn–Hägerdal, 2000; Auras *et al.*, 2004). Most homofermentative strains of LAB can convert glucose to lactic acid, including *Lactobacillus delbrueckii*, *Lactobacillus helveticus*, and *Lactobacillus acidophilus*. Contrarily, heterofermentative LAB are capable of utilizing both hexoses and pentoses. For example, *Lactobacillus pentosus* ATCC 8041 was used to convert hydrolysates of trimming vine shoots (Bustos *et al.*, 2004) and corn cobs to lactic acid (Zhu *et al.*, 2007). *Lactobacillus bifermentans* DSM 20003 was also reported to convert a mixture of glucose, arabinose and xylose into lactic acid (Givry *et al.*, 2007). *Lactobacillus brevis* ATCC367, a well-known heterofermentative strain, was reported to metabolize sugars such as glucose, xylose and lactose (Cui *et al.*, 2011).

Normally, LAB may suffer some limitations as production of both L- and D-lactic acid and D-lactate dehydrogenase, low yield, due to byproducts formation, and high risk of cell lysis by bacteriophage infection (Abdel-Rahman *et al.*, 2013; Talukder *et al.*, 2012).

Mixed cultures or co-cultures in fermentation may provide useful combinations of metabolic pathways for the utilization of complex materials and, consequently, enhance lactic acid production (Cui et al, 2011). For example Nancib et al. (2009) compared lactic acid production from date juice by single and mixed cultures of *Lactobacillus casei* and *Lactococcus*

lactis. They reached an efficiency of 96% based in glucose utilization with mixed cultures after 19-h incubation. Other mixtures have already been studied with good results for lactic acid production as *Lactobacillus casei* and *Lactobacillus delbrueckii* in the fermentation of cassava bagasse via SSF (John *et al.*, 2006) and the co-culture of *Enterococcus casseli flavus* and *Lactobacillus casei* (Taniguchi *et al.*, 2004).

Genetic-engineering approaches have been strongly exploited for the improvement of lactic acid yield and optical purity by various microbial producers. As an example, the improvement of optical purity via the deletion of either D- or L-lactate dehydrogenase (LDH) genes (Kylä-Nikkilä *et al.*, 2000), increased lactic acid yields through reduction of byproduct levels by the deletion of different genes, development of bacterial strains, e.g., *Escherichia coli*, and strain improvements for blocking steps in phage life cycle (Gaspar *et al.*, 2013).

1.1.3 Metabolism

Among lactic acid-producing bacteria there are wild-type and engineered producers including lactic acid bacteria (LAB), *Bacillus* strains, *Escherichia coli* and *Corynebacterium glutamicum* (Abdel-Rahman et al., 2013). Lactic acid has been traditionally produced by lactic acid bacteria (LAB). LAB ferment sugars to lactic acid via different pathways, homofermentation, heterofermentation and mixed acid fermentation (Hofvendah and Hahn-Hägerdal 2000).

Homofermentative bacteria produce lactic acid as the major end product and use the Embden–Meyerhof–Parnas pathway (Hofvendah and Hahn-Hägerdal 2000; Abdel-Rahman et al., 2013). Glucose is transformed into pyruvic acid, while in the latter this is reduced to lactic acid (Figure 1-2). Theoretically, the yield of fermentation is 2 mol of lactic acid per mole of consumed glucose (1 g of product per g of substrate), however because of the use of a portion of the carbon source for biomass production, the experimental yields are usually low (0.74-0.99 g/g) (Castillo Martinez *et al.*, 2013).

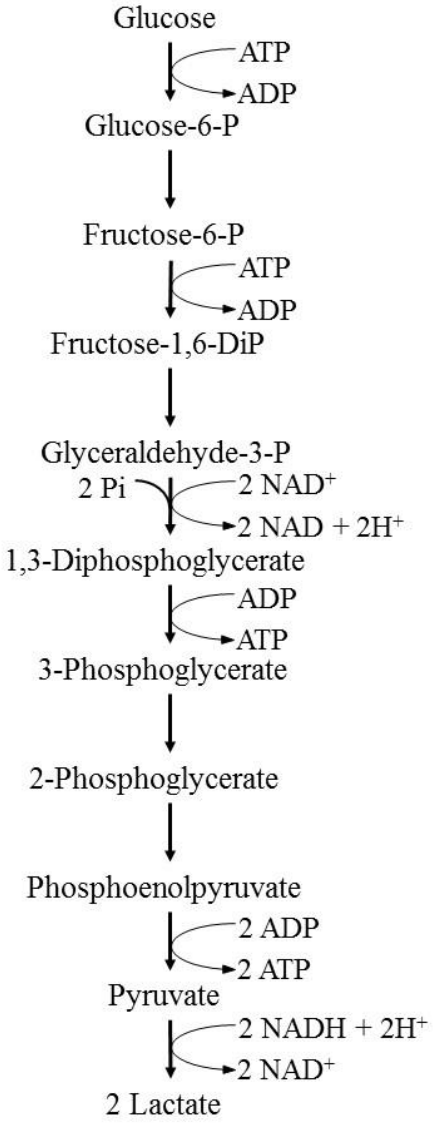

Figure 1-1 Homofermentative pathway for lactic acid production

Heterofermentation process is characterized by the formation of co-products, other than lactic acid, such as CO_2, ethanol and/or acetic acid. The maximal yield of lactic acid per glucose is 1.0 mol/mol or 0.5 g of lactic acid per g of substrate (Abdel-Rahman et al., 2011). This process use the alternate pentose monophosphate pathway (Figure 1-3), converting 6-carbon sugars (hexoses) to 5-carbon sugars (pentoses) by phosphoketolase (Abdel-Rahman et al., 2013).

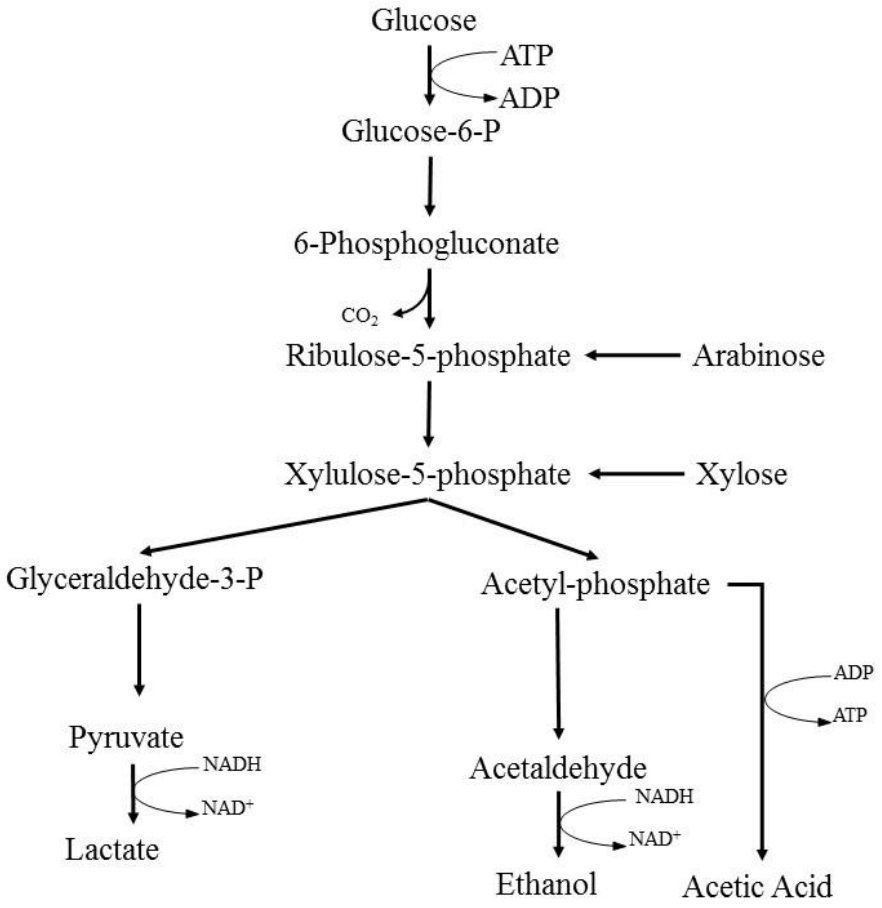

Figure 1-2 Heterofermentative pathway for lactic acid production

Homofermenters can form ethanol, acetic acid and formic acid, only under glucose limitation, during growth with consumption of other sugars (maltose, lactose and galactose), or variations of pH (increase) and temperature (decrease) (Hofvendah and Hahn-Hägerdal 2000).

LAB requires complex nutrients due to their limited ability to synthesize B-vitamins and amino acids. *Escherichia coli* has been used for lactic acid production because *Escherichia coli* does not require a complex medium and can grow in a simple mineral salt medium. However, the yield of lactic acid needs to be improved, then, genetic modifications of *Escherichia coli* has been used for this purpose (Hofvendah and Hahn-Hägerdal 2000). Wang et al. (2012) studied genetic modification for improvement of D-lactic acid production in *Escherichia coli*, resulting in 85 g/l of D-lactic acid from 100 g/l of glucose. The strain was engineered by chromosomal deletion of the competing fermentative pathway genes (adhE, frdABCD, pta, pflB, aldA) and the repressor gene (cscR) of the sucrose operon, and metabolic evolution for improved anaerobic cell growth. Production of L-lactic acid by engineered *Escherichia coli*

was studied using different substrates such as glycerol (Mazumdar *et al.*, 2013) and xylose (Zhao *et al.*, 2013).

1.1.4 Recovery processes

The separation and purification of lactic acid, and many other biotechnological products, is a crucial industrial step, either from the economical point of view – considering in some cases the downstream processing accounts for more than 50% of the overall operational costs – and also from technical point of view, since, depending on the case, high purities of the product are desired and loss of product must be avoided.

The classical separation steps involve conventional filtration followed by the application of high cost chemicals, with generation of undesired products (like gypsum) (Chaudhuri and Phyle, 1992; Eyal and Bressler, 1993).

Recently, several other alternatives have been proposed and/or adapted for lactic acid separation and purification, and depending on the objectives can present advantages and disadvantages. The most important ones are: distillation, esterification, nano and ultrafiltration, liquid-liquid extraction, adsorption, ion exchange chromatography and electrodialysis.

Distillation of solutions containing lactic acid is an alternative, but has some important drawbacks, like the difficulty to separate lactic acid from other organic acids, and the high boiling point of lactic acid (around 122^{o}C at 12 mmHg). Moreover, in this temperature the water content tends to be drastically reduced, and the lactic acid tends to self polymerize. In this context, the esterification of lactic acid with alcohols prior to the distillation of the products (lactate esters), followed by hydrolysis and recovery of lactic acid, is an interesting alternative, allowing its separation from other organic acids. The main technical difficulty concerning esterification is the thermodynamic equilibrium.

In order to shift the reaction toward the products of esterification, some strategies can be adopted, like the use of excess of ethanol, concomitant distillation or the use of bipolar membranes. Bipolar membranes allow the products of esterification to be separated from the reactants by means of diffusion, which is not dependant from other physicochemical characteristics of the involved compounds, like boiling point or vapor pressure, and has gained notability in recent works (Khunnonkwao *et al.*, 2012).

About liquid-liquid extractions, the classical extractants are tri-n-butylphosphate (TBP), tri-n-octylamine (TOA) and tri-n-octylphosphineoxide (TOPO), which can be used in pure form or mixed with other solvents, like toluene, hexanol and octanol. These classical extractants give

good yields (good selectivity coefficients) when lactic acid is present in high concentrations in the culture broth.

Recently, ionic liquids have been used as extractants. Ionic liquids have particular properties, like low vapor pressure and molecular structure with polar and apolar regions, which puts them as an alternative for specific purposes in an extraction process, enabling in some cases better selectivity, substrate solubility and product separation. Some good results (very high selectivity coefficients) have been obtained for broths with low concentrations of lactic acid (lower than 0,2 M) with ionic liquids as extractants. For example, Martak et al. (2008) obtained partition coefficients higher than 40 for lactic acid, using phosphonium-based ionic liquids.

The technique of adsorption has been successfully employed for purification of lactic acid. A common strategy is to develop silica compounds with functional groups. The challenge is to research and develop the most suitable ligands considering their affinity with lactic acid. The main advantage of this strategy when compared to liquid-liquid extraction is avoiding leaching of the extractive solvent to the aquous phase (Krzyżaniak *et al.*, 2013).

Ion exchange chromatography is an extremely selective method of separation, and can provide very high product recovery yields. An example is the separation of lactic acid from a grass silage juice using a neutral polymeric resin, achieving purities from 93.2 to 99.9% and recovery yields up to 97% (Thang and Novalin, 2008).

About the electrodialysis, the efforts are mainly to establish a process capable to separate the lactate salts in a concomitant fermentation process, which would allow a higher process yield, avoiding the accumulation of lactate in the culture broth and would diminish the effect of product feedback regulation over lactate production. However, the drawback of this alternative is the damage caused to the cells by the electrodes. To solve this issue, one interesting option is the use of ultrafiltration (Nomura *et al.*, 1991). With this strategy, the cells will not be in contact with the electrodes. Another issue is pH change during fermentation. Besides the obvious alternative of using alkaline compounds, the integration of electrodialysis and water bipolar membranes have allowed the separation of lactate salts in lactic acid and base – the lactic acid can be separated and the base can be reused to control the pH (Li *et al.*, 2004).

1.1.5 Demand and products

Due to its versatility as basic input and large amount of application are found in the food, textile, chemical and pharmaceutical industry, like acidulant, preservative and

intermediate for many final products. According to Yadav et al. (2011) and Jamshidian et al.(2010), lactic acid world demand is increasing every year at a rate estimated at 5-8%, with a production projected to 2017 around 370,000 metric tons. The fermentative pathway is gaining importance for lactic acid production, due to the quantity of research and a broad range of microorganisms are able to produce lactic acid from many alternative substrates, much of them industrial residues, with less-expensive final products.

L-lactic acid is the most common commercial form because the human body is better adapted to assimilate it. L-lactic acid is also polymerized to a highly crystalline PLA, suited for commercial use, in fiber and film production (Connolly *et al.*, 2005; Södergård and Stolt, 2002). The development of new polymers such as the stereocomplex PLA, a highly thermostable polymer composed of both L- and D-lactic acid monomers, has attracted new interest to D-lactic acid production (Okano *et al.*, 2009; Yoshida *et al.*, 2011).

Lactic acid polymerization is also gaining importance due to the polymer characteristics, biocompatibility to produce human prosthesis to replace bones and approval to be used in food package.

Both, lactic acid and polylatic acid, are produced worldwide by many enterprises in many countries such as: USA: Nature Works LLC, leader in PLA technology, Cereplast Inc.; The Netherlands: Purac, Hycail; Belgium: Futerro, Galactic; China: Hisun Industries Co. Ltd, Snamprogetti; Germany: Biomer Technology Ltd, Stanelco RF Technologies, Uhde Inventa-Fischer; Japan: Toyobo, Dai Nippon Printing Co., Ltd., Mitsui Chemicals, Inc., Shimadzu Corporation, NEC Corporation, Toyota Motor Corporation, in different capacities and using different technologies (Jamshidian et al., 2010).

PLA is expected to replace fossil fuel-based plastics in many applications, but to be competitive; PLA's production cost must decrease to a half of its present price. The bottleneck of the PLA price is the monomer high fermentative production costs. This includes costs with the substrate, nitrogen sources, with the recovering and purification process. It is very important to search for more productive microorganisms strains to improve operating process efficiency and yields of lactic acid production along with the necessity of a high concentrate lactic acid solution to improve the polymerization process (Lopes et al., 2012; Abdel-Rahman et al., 2013).

1.2 POLY(LACTIC ACID) (PLA)

1.2.1 Properties of PLA

The monomer lactic acid has two optical isomers, L and D, resulting in polymers that can present three forms: PLLA, PDLLA and PDLA. The stereochemistry, the ratio of L and D isomers, influences on PLA properties and degradability (Abdel-Rahman *et al.*, 2011). The optical purity of PLA has effect on the crystalinity of the polymer.

PLAs with L-content (PLLA) greater than 90% tend to be crystalline while those with lower optical purity are amorphous. The melting temperature (Tm), glass transition temperature (Tg), and crystallinity decrease with decreasing amounts of L-isomer (Lasprilla *et al.*, 2012). Physical characteristics depend on the transition temperatures, such as mechanical and rheological characteristics, density and heat capacity (Henton *et al.*, 2005; Lim *et al.*, 2008). PLA has lower Tm and Tg than poly(ethylene terephthalate) (PET) and polystyrene (PS), which make PLA better for heat sealing and thermal processing. PLA is a clear, colorless thermoplastic when quenched from the melt and it is similar in many aspects to PS (Auras *et al.*, 2003; Madhavan Nampoothiri *et al.*, 2010).

Mechanical properties of PLA can vary according to different parameters, such as crystallinity, polymer structure and molecular weight, material formulation (plasticizers, blend, composites, etc.) and processing. It can range from soft and elastic materials to stiff and high strength materials (Averous, 2008). PLA has properties comparable to existing petroleum-based polymers such as high strength, modulus and transparency, etc. (Lim *et al.*, 2008; Liu *et al.*, 2011; Carrasco *et al.*, 2010). However, it features brittle behavior and among various modifications, melt blending is the most economic means to improve its toughness (Liu *et al.*, 2011). PLA films (containing more than 94% of L-lactide) show good tensile strength with higher values than PS but lower than PET (Auras *et al.*, 2003). Thermal and mechanical properties of PLA are higher than the other biodegradable polyesters (polybutylene succinate - PBS, polyhydroxybutyrate - PHB and polycaprolactone– PCL) (Brito *et al.*, 2011).

Gas permeation properties of PLA are important for application in the packaging industry. The permeation of gases in polymers depends on factors, such as polymer-gas molecule chemistry, free volume in the amorphous phase and crystallinity (Bao *et al.*, 2006). The permeation properties of small gases are not affected by changes in polymer chain branching and L:D ratios (Lehermeier *et al.*, 2001; Auras *et al.*, 2003; Bao *et al.*, 2006). Lehermeier et al. (Lehermeier *et al.*, 2001), the permeation properties of PLA to some gases, O_2, CO_2, N_2 and CH_4, which are very similar to polystyrene. Auras et al. (Auras *et al.*, 2003) studied the permeability of PLA to O_2, CO_2 and water vapor. PLA showed lower CO_2

and O_2 permeability coefficients than PS and comparable to those of PET and their water vapor permeability coefficients do not vary significantly with relative humidity.

The solubility of PLA varies according to its stereochemistry form, besides it is not soluble in water, methanol, ethanol, propylene glycol, hexane and heptane (Madhavan Nampoothiri *et al.*, 2010). PLLA is soluble in chloroform, furan, dioxane and dioxolane and it is not soluble in acetone, ethyl acetate or tetrahydrofuran (Madhavan Nampoothiri *et al.*, 2010). PDLLA is soluble in chloroform, furan, dioxane, dioxolane and acetone, and PDLA in ethyl lactate, tetrahydrofuran, ethyl acetate, dimethylsulfoxide, xylene and dimethylformamide (Lasprilla *et al.*, 2012).

1.2.2 Synthesis of PLA

1.2.2.1 Methods of polymerization

The polymerization of PLA requires the monomer of high purity, because the impurities can affect the reaction reducing the rate of polymerization resulting in a low quality and low molecular weight polymer (Gupta and Kumar, 2007). PLA can be obtained by two different ways: direct polycondensation of LA and ring-opening polymerization (ROP) of lactide (Figure 1-4). The polycondensation method generally prepares a low molecular weight PLA, thus, a high molecular weight can be obtained by azeotropic condensation polymerization and solid-state polymerization (Madhavan Nampoothiri *et al.*, 2010; Vouyiouka *et al.*, 2013).

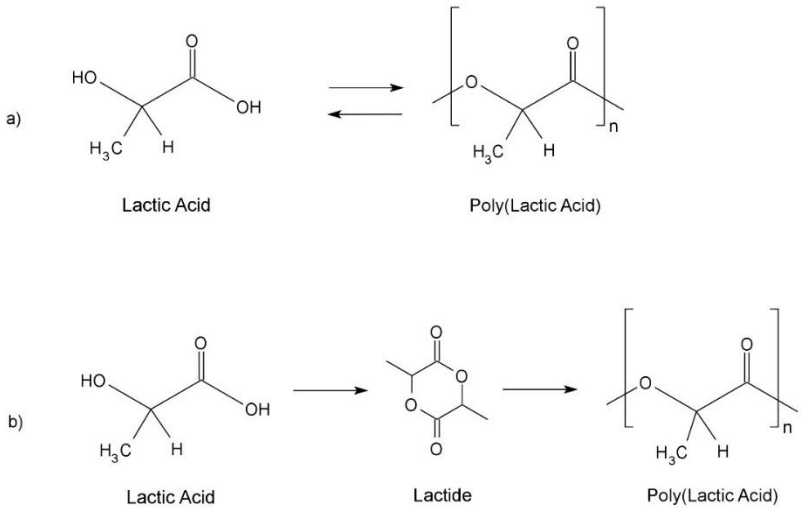

Figure 1-3 Lactic acid polymerization methods to obtain PLA. a) Direct polycondensation and b) ring opening polymerization

Direct polycondensation polymerization

According to Södergärd and Stolt (2010), the three main steps of PLA direct condensation are: removal of the free water content, otherwise it can form some low amount of oligomers (linear dimer, linear trimer, etc.) in this stage. In the second step, oligomerization, in which the LA is converted into low molecular weight PLA. The low molecular weight PLA reaction can be carried out using catalysts such as strong acids and organometallic compounds, using an evaporator or a stirred reactor. However, it can also occour side reactions at temperatures above 120 ºC (Madhavan Nampoothiri *et al.*, 2010). The third stage is the melt polycondensation, where the removal of water becomes critical from the highly viscous reaction mixture. It is essential to ensure the water withdrawal to obtain high molecular weight polymers. The water formed in the reaction can enhance a hydrolysis reaction of the formed polymer (Gupta and Kumar, 2007; Marques *et al.*, 2010; Södergärd and Stolt, 2010).

Some studies reported the production of high molecular weight PLA by direct polymerization. Chen et al. (Chen *et al.*, 2006) observed, high molecular weight of PLA contrary to the common expectations. In their study a PLLA polymer of 120,000 g/mol was obtained using titanium(IV) butoxide as catalyst under nitrogen atmosphere and vacuum. The duration of the decompression of the reaction pressure to 1 Torr was important. Achmad et al. (Achmad *et al.*, 2009) obtained 90,000 g/mol PLA using L-LA 60% (w/w) under vacuum without catalyst and solvent in order to reduce the production costs, nevertheless the time required was very long, 96 h.

High molecular weight PLA can be synthetized azeotropically and by solid state polymerization. In the azeotropic synthesis, an organic solvent is used for azeotropic distillation of condensation water. This technique uses a high activity catalyst and a low boiling organic solvent. The water formed during the synthesis is removed azeotropically, while the solvent is treated with a drying agent and then recycled back in the reaction. The reaction temperature used is below the melting temperature of the polymer and prevents depolymerization and racemization during polymerization (Madhavan Nampoothiri *et al.*, 2010; Gupta and Kumar, 2007; Enomoto *et al.*, 1994).

A general procedure for azeotropic synthesis consists in the distillation of LA during 2-3 h at 130°C with organic solvent (Table 1-2) and catalyst at reduced pressure to remove most of the condensation water in a Dean Stark trap. After, the solvent returns to the vessel by molecular sieving during 30−40 h at 130°C (Averous, 2008; Ajioka *et al.*, 1995; Garlotta, 2001). Berger and Gregorova (Berger and Gregorova, 2014) synthesized PLA by azeotropic condensation of L-LA in xylene. Moreover, functional end groups of PLA were modified by

succinic anhydride and l-cysteine by the addition–elimination reaction. Yamada et al. (Yamada et al., 2014) investigated the polymerization of LA in xylene catalyzed by scandium trifluoromethanesulfonate using a Dean–Stark apparatus under a conventional heating process and microwave heating process. The molecular weight of the obtained polymer was higher using microwave heating process.

Table 1-2 Organic solvents used in azeotropic polymerization

Hydrocarbon solvents	Toluene, xylene and mesitylene
Halogenated hydrocarbon	Chlorobenzene, bromobenzene, iodobenzene, dichlorobenzene, 1,1,2,2, -tetrachloroethane and p-chlorotoluene
Ketone solvents	3-hexanone, acetophenone and benzophenone
Ether solvents	Dibutyl ether, anisole, phenetole, o-dimethoxybenzene, p-dimethoxybenzene, 3-methoxytoluene, dibenzyl ether, benzyl phenyl ether and methoxynaphthalene
Thioether solvents	Phenyl sulfide and thioanisole
Ester solvents	Methyl benzoate, methyl phthalate and ethyl phthalate; diphenyl ether; alkylated diphenyl
Alkylated diphenyl ether solvents	4-methyldiphenyl ether, 3-methyldiphenyl ether and 3-phenoxytoluene;
Halogenated diphenyl ether solvents	4-bromodiphenyl ether, 4-chlorodiphenyl ether and 4-methyl-4'-bromodiphenyl ether; alkoxy diphenyl ether solvents such as 4-methoxydiphenyl ether, 3-methoxydiphenyl ether and 4-methyl-4'-methoxydiphenyl ether
Cyclic diphenyl ether solvents	Dibenzofuran and xanthene.

In solid state polymerization (SSP), after the melt polycondensation process, the formed prepolymer is cooled followed by particle formation with relative low molecular weight in powder, pellet, chip or fibre form. The solid polymer is heated at temperatures between Tg and Tm under inert atmosphere (Gupta and Kumar, 2007; Vouyiouka *et al.*, 2013; Vouyiouka *et al.*, 2005). It is possible to observe in the prepolymer a crystalline region and an amorphous region where the reaction of SSP occurs because the chain mobility is high enough, furthermore end groups, low molecular weight substances and catalysts are concentrated in the amorphous phase (Vouyiouka *et al.*, 2005; Södergärd and Stolt, 2010). Metal catalyst such as Sn, Ti and Zn can catalyze the SSP in the amorphous phase besides the melt polycondensation (Södergärd and Stolt, 2010; Vouyiouka *et al.*, 2013).

SSP has advantages such as control of thermal, hydrolytic, oxidative degradations because of low operating temperature, reduced discoloration, degradation of the product and no

environmental pollution, because no solvent is required. Improved properties of polymers are reached due to the limit of monomer cyclisation and other side reactions (Gupta and Kumar, 2007). Pivsa-Art et al. (Pivsa-Art *et al.*, 2013) synthetized PDLA by 2 steps direct polycondensation comprised of melt polymerization and SSP using D-lactic acid as monomer catalyzed by 2- Naphthalenesulfonic acid. In this case, the obtained molecular weight was 33,300 Da. Vouyiouka et al. (Vouyiouka *et al.*, 2013) in a fixed bed reactor under nitrogen flow, submitted PLA to SSP, resulting in an increase of molecular weight up to 1.7 times. A nanocomposite poly (L-lactic acid)-clay obtained by SSP was studied by Katiyar and Nanavati (Katiyar and Nanavati, 2011).

Ring Opening Polymerization (ROP)

In this process, PLA is obtained from a lactide (3,6 dimethyl 1,4-dioxane 2,5-dione) (Figure 1-5), a cyclic lactic acid dimer, present in three stereoisomeric forms DD-, LL- and DL(meso)-lactides (Gupta and Kumar, 2007), which is formed in the first step when the condensation product water is removed by evaporation during oligomerization. The low molecular weight PLA oligomer is submitted to a thermal cracking at high temperature and low pressure in the presence of catalyst and prepares the lactide (Madhavan Nampoothiri *et al.*, 2010; Gupta and Kumar, 2007). In order to control the final PLA structure, the separation between each stereoisomer can be done based on the boiling point differences (Averous, 2008).

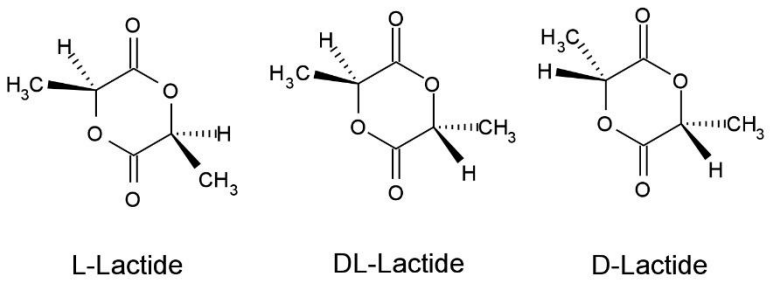

Figure 1-4 Lactide streoisomeric forms

The ROP is applied commercially, however this is a high cost process due to the purification process of the lactide. Impurities can deteriorate mechanical properties, cause corrosion of the processing machines and increase polymer degradation rate (Shinno *et al.*,

1997; Vouyiouka *et al.*, 2013). High molecular weights were not obtained until improved lactide purification techniques were developed by DuPont in 1954 (Averous, 2008).

ROP has different reaction mechanisms such as ionic (anionic or cationic) or coordination–insertion, depending on the catalytic system (Gupta and Kumar, 2007; Averous, 2008). The most widely studied method for the synthesis of high molecular weight PLA is coordination–insertion. In this method, catalysts like metal alkoxide are used such as Mg, Sn, Ti, Zr, Zn, which have a covalent bond between metal atom and oxygen atom and behave like weak Lewis acids (Stolt and Södergård, 1999; Averous, 2008; Gupta and Kumar, 2007). Typical conditions for polymerization are 180°–210°C, tin octoate as catalyst at concentrations of 100–1000 ppm, and 2–5 h. In these conditions, it reaches 95% conversion. The polymerization is first order in both catalyst and lactide. Hydroxyl-containing initiators, such as 1-octanol, can be used to both molecular weight control and reaction acceleration (Henton *et al.*, 2005). Studies with ROP are shown in Table 1-3.

Table 1-3 Ring opening polymerization (ROP) for PLA synthesis

Monomer	Catalyst	Condition	Mw	Reference
D,L-lactic acid	tin(II) chloride - polycondensation tin octoate	170 ºC and 120 torr for 3 hours for polycondensation. 220 ºC and 60 torr for lactide production. 130 ºC for 24 hours for ROP.	19,264	(Orozco et al., 2014)
L-lactide	Dibutyltindimethoxide	Nitrogen atmosphere, 130 ºC for 40 h	7,896	(Singla et al., 2012)
L-lactide	Zinc stearate	Nitrogen atmosphere, 130 ºC for 65 h	19,124	(Singla et al., 2012)
L-lactide	Dibutyltindimethoxide	Under vacuum, 130 ºC for 133 h	3,460	(Singla et al., 2012)
L-lactide	Zinc stearate	Under vacuum, 130 ºC for 34.5 h	27,534	(Singla et al., 2012)
L-lactide	Salen catalyst Fe	Glass ampoule sealed under vacuum at 200 ◦C	34,000	(Idage et al., 2010)
L-lactide	Salen catalyst Mn	Glass ampoule sealed under vacuum at 200 ◦C	25,000	(Idage et al., 2010)
L-lactide	tin octanoate/toluene	Glass ampoule sealed under vacuum at 160° C for 2 h/ SSP at 150° C for 10 h	108,500	(Nanavati and Katiyar, 2010)
L-lactide	tin octanoate-oligomer-lactide catalyst complex	Under vacuum at 160° C for 1 h/SSP at 150° C for 10 h	228,000	(Nanavati and Katiyar, 2010)

L-lactide	tin octanoate / octanol	supercritical carbon dioxide, 3500 psi at 80 ºC, 47 h using poly-(dimethylsiloxane)s	14,000	(Ganapathy et al., 2007)

Enzyme-Catalyzed Polymerization

Living organisms produce macromolecules for their metabolism such as polysaccharides, polynucleotides, proteins, or polyesters. The polymerization reaction occurs in vivo catalyzed by enzymes and the monomers are usually formed by complex metabolic process. The interesting for the use of enzymes has increased, moreover the use for polymerization reaction in vitro (Gross *et al.*, 2001). Enzymatic polymerization is an in vitro chemical polymer synthesis catalyzed by an isolated enzyme (nonbiosynthetic or nonmetabolic pathways) (Kobayashi *et al.*, 2001). Poly(hydroxyalkanoate)s (PHAs), a biodegradable polyester, were produced by fermentation in the 1980s. In vitro synthesis of polyesters has been developed since the 1980s using a lipase enzyme catalyst (Kobayashi and Makino, 2009).

In vivo enzymatic catalysis has specific characteristics. There are significant differences between enzyme-catalyzed processes. All in vivo reactions occurs under mild conditions, in most cases in water at neutral pH value and lower temperature. However, in vitro enzyme catalyzed reactions are generally performed non-aqueous solvents and the properties of enzymes are different in organic solvents whose is distinct from water. Enzyme enantioselectivity depends on the method of preparation and the temperature of reaction in different organic solvents (Varma *et al.*, 2005).

Some lipases are stable in organic solvents and can be used as catalyst for esterification and transesterification depending on the used solvent system, they can be applied for hydrolysis reactions or ester synthesis. This specific catalysis enables the production of useful polyesters and polycarbonates by various polymerization modes, such as polycondensation and ring opening polymerization. Many condensation reactions that are difficult or impossible in aqueous media are catalyzed by lipases in non-aqueous media. (Varma *et al.*, 2005)

Different enzymes were employed for LA polymerization. Porcine Pancreas Lipase (Sigma Chemical Company), Lipozyme IM20, Rhizomucor miehei (Novo Nordisk) and Chirazyme and lipase (Boehringer Mannheim), catalyzed the reaction for a long period of time (10-30 days) in different solvents such as hexane–chloroform, hexane–methyl isobutyl ketone and benzene. The highest molecular weight was obtained using porcine pancreas lipase in solvent hexane–methyl isobutyl ketone and benzene., It was achieved a yield of 80.1 % and 3300 of molecular weight when using succinic acid. Blends of enzymatically prepared

polylactic acids with polystyrene yielded very good films in terms of tensile strength, elongation and optical properties (Kiran and Divakar, 2003).

Distel et al. (Distel *et al.*, 2005) improved the solubility of enzymes in organic solvents for their use as biocatalyst in PLA preparation. The enzymes Proleather (an alkaline protease from Bacillus sp.) and Novozyme-435 (lipase B from Candida antarctica) were submitted to deanoyl chloride, a modifier of enzyme solubility. Proleather enzyme catalyzed transesterification reactions faster than the lipase-catalyzed polycondensation polymerization of ethyl lactate to PLA. The modified enzyme has highest enzyme activities with THF and chloroform, however in acetone in presented lowest enzyme activity.

Microwave heating process

Microwave (MW) heating process is a tool for chemical reactions, heating and drying materials utilized in many private households and industries. MW has high potential to strikingly accelerate the chemical reaction and drastically reduce the production energy, when compared with the conventional conductive heating processes. Furthermore, it reduces side reactions, increases yields and improves reproducibility (Kappe, 2004). The first experiments in microwave were performed in domestic, sometimes modified kitchen microwave ovens. Then, specialized instruments with temperature and pressure control have been current used. MW can directly heat materials via a dielectric interaction between materials (Nakamura *et al.*, 2010). Molecules exhibiting a permanent dipole moment will align to the applied electromagnetic field generating heat because of friction, and collision of molecules (Hoogenboom and Schubert, 2007). The high temperatures attained and the ability to work under high pressure conditions for relatively short times make reactions faster than under conventional thermal conditions and greater yields are usually obtained (Kappe, 2004; Sosnik *et al.*, 2011).

The polycondensation of lactic acid by MW irradiation is drastically accelerated in the gram-scale reaction size. However, there were some difficulties to estimate the energy efficiency in the MW reaction because of the small size of the reaction (Nakamura *et al.*, 2010). Some studies indicated that there was degradation of PLA during microwave heating process. The increase of irradiation time causes the decreasing of the yield of polycondensation due to the loss of oligomers of lower mass during the reaction (Kéki *et al.*, 2001). Irradiation time and decrease in the amount of high boiling point solvent may produce higher viscosity products (Watanabe *et al.*, 1993). The water produced during the reaction must be removed in order to avoid degradation of polymer. A reduction of pressure can be used to remove the water

produced. However, at low pressure, 30 mmHg, the molecular weight started to decrease due to losses of monomer and other volatile oligomers (Nagahata *et al.*, 2007). Degradation of the reactants can be caused by microwave plasma formed at pressures lower than 3000 Pa (Nakamura *et al.*, 2010). MW has been widely used for direct polycondensation and ring-opening polymerization. Few reports have been found in the literature concerning microwave-irradiation to produce PLA (Table 1-4).

Table 1-4 Microwave (MW) heating process for PLA synthesis

Monomer	Catalyst	Power (W)	Temperature (ºC)	Pressure (Pa)	Time (minutes)	Molecular Weight	Reference
LA (85%)	Sc(OTf)3 / xylene	300	160	N	360	11,600	(Yamada *et al.*, 2014)
L-lactide	Sn(Oct)2 / Diethyl ether	180	180	Bottle sealed under vacuum	10-30	10,000	(Singla *et al.*, 2012)
LA 90%	SnCl2 / p-TsOH	200 - 700	180	1000	240	10,000	(Nakamura *et al.*, 2010)
LA 90%	SnCl2 / p-TsOH	200 - 1400	180	100	240	10,000	(Nakamura *et al.*, 2010)
LA 90%	SnCl2 / p-TsOH	1000 - 6000	180	800	225	10,000	(Nakamura *et al.*, 2010)
LA 85%	SnCl2 / P-TsOH	40	200	4000	30	16,000	(Nagahata *et al.*, 2007)
L-LA 85%	Sn(Oct)2	130 - 400	207	93325	135	30,000	(Pandey and Aswath, 2009)
LA	Al2O3/SO4	50	260	2000	60	20,000	(Cao *et al.*, 2009)
DL-lactide	Sn(Oct)2	450	N	Atmospheric Pressure	30	200,000	(Jing, Peng, Tong, *et al.*, 2006)
LA 85%	N	320	N	5000 – 40000	35	4,000	(Jing, Peng, Yingmin, *et al.*, 2006)
Olygomer	SnCl2 / TSA	528	N	5000	40	50,000	(Jing, Peng, Yingmin, *et al.*, 2006)
LA 85%	Sn(Oct)2	120	140-160	2500	270	7,030	(Jiménez-Bonilla *et al.*, 2014)

1.2.3 Applications of PLA

PLA meets many requirements as a packaging thermoplastic and is suggested as a commodity resin for general packaging applications. Owing to biodegradability,

biocompatibility, thermoplastic processability and eco-friendliness, it offers potential applications in packaging, agricultural products, medical and textile industry. Furthermore, PLA has been utilized as medical material and drug delivery systems (Gupta and Kumar, 2007; Madhavan Nampoothiri *et al.*, 2010; Lasprilla *et al.*, 2012).

1.2.3.1 Medical applications

For medical applications, biodegradable materials have been studied extensively, due to their advantages over non-degradable biomaterials including eliminate a secondary operation to remove them after the defect site is repaired and providing long term biocompatibility (Suzuki and Ikada, 2010; Lasprilla *et al.*, 2012). Due to its relatively strong mechanical properties, PLA has been used in many medical implants and it is approved by regulatory agencies in many countries. PLLA is a semicrystalline material and its degradation occurs after more than 2 years. PDLLA is amorphous and degrades within 16 month (Middleton and Tipton, 2000; Suzuki and Ikada, 2010).

Tissue engineering has utilized biodegradable polymers to build scaffolds, three-dimensional porous materials, necessary support for cells regeneration of tissue. It combines cells, biomaterials, and micro-environmental factors to induce differentiation signals into surgically transplantable formats and promote tissue repair and/or functional restoration (Malafaya *et al.*, 2007). Several scaffold materials have been investigated including hydroxyapatite (HA), poly(α-hydroxyesters), and natural polymers such as collagen and chitin (Hutmacher, 2000).

Biomaterials such as PLA and poly(glycolic acid) (PGA) were required because of suitable mechanical properties closely matched to the target tissues and good biological interaction with cells (Cai *et al.*, 2002). Deng et al. (Deng *et al.*, 2014) tested the effect of adipose-derived stem cells (ASCs) on engineered tendon repair in vivo using rabbit Achilles. The scaffold was composed by polyglycolic acid (PGA) and polylactic acid (PLA) fibers as the scaffold in a ratio of 4:2 to provide essential mechanical strength. In vitro culture of rabbit ACSs were seeded evenly on to the composite scaffold to form cell-scaffold constructs. Afterwards, culture medium was added, and the cell-scaffold construct was incubated in a bioreactor for in vitro culture during 5 weeks with mechanical loading. Then, in vitro cultured constructs prior to in vivo transplantation. Cell-seeded constructed gradually form neo-tendon and became more mature at 45 weeks with histological structure similar to that of native tendo. In contrast, cell-free constructs failed to form good quality tendon tissue with fibril structure observable only at 45 weeks. A biphasic scaffold, consisted of (PGA/PLA) scaffold and poly-ε-

caprolactone/hydroxyapatite (PCL/HA) scaffold, was designed and used by Ding et al. (Ding *et al.*, 2013) for regeneration of goat femoral head. In the study, chondrocytes were seeded into the PGA/PLA scaffolds cultured *in vitro*. After implantation in nude mice subcutaneously, the cell–scaffold constructs successfully regenerated goat femoral heads.

One great limitation of PLLA is the lack of compatibility for cells due to its hydrophobicity. One approach to solve this problem is to immobilize a biocompatible layer on the surface of the polymer to improve cell-material interactions (Ma *et al.*, 2002). Cai et al. (Cai *et al.*, 2002) studied the modification the surface of PDLLA film with silk fibroins, a naturally occurring structural protein, in order to increase the interaction between PDLLA films and osteoblasts. Rat osteoblasts were cultured in vitro on the modified PDLLA films. The modification of PDLLA with silk fibroins improved the hydrophilicity of films and the presence of nitrogen atoms was confirmed. The modified film with coverage of 65% of silk fibroins was most suitable for osteoblast culture, which indicated that the amount of the anchored SF was not necessarily correlated to cell culture. In order to improve the hydrophilcity and cell compatibility of the scaffolds for tissue engineering applications, Oh et al. (Oh *et al.*, 2003) fabricated a blend between poly(lactic-*co*-glycolic acid) (PLGA) and polyvinyl alcohol (PVA). A suspension of chondrocytes was seeded to the each scaffold in vitro culture. The scaffolds were implanted in rabbits for in vivo tissue compatibility evaluation.

1.2.3.2. Drug Delivery System

Modified drug delivery system improves efficacy, reduces toxicity, improves patient compliance and convenience if compared to conventional dosage forms (Uhrich *et al.*, 1999). Moreover, improves bioavailability and maintains drug concentration by controlling the drug release rate (Zhang *et al.*, 2013). The mechanism can occur by temporal control or distribution control. Delivering the drug over an extended duration or at a specific time during treatment is temporal control mechanism. Distribution control involves targeting the release of the drug to the precise site of activity within the body. A large number of classes of drugs can be benefit such as chemotherapeutic drugs, immunosuppressants, antiinflammatory agents, antibiotics, opioid antagonists, steroids, hormones, anesthetics, vaccines, peptide drugs and gene therapy (Uhrich *et al.*, 1999).

PLA, PGA and their copolymer, poly(lactide-co-glycolide) (PLGA) are the most widely used polymers in drug delivery system development, because of their biodegradability, biocompatibility and ease of processing (Zhang *et al.*, 2013). Physical characteristics of PLGA, such as size particle, can be controlled by synthesis method Employed and changing the

chemical composition (Lü *et al.*, 2009; Zhang *et al.*, 2013). PLA polymer became interesting due to the degradation product is lactic acid, a metabolism product of organisms. Preventive retinal degeneration was studied by Rafat et al. (Rafat *et al.*, 2010) due to the restricted permeability of the corneal and conjunctival epithelia and the presence of the blood-retina barrier is a challenge for conventional methods to drugs delivery to the retina. The authors evaluated the prevention of retinal degeneration using a transactivator of transcription-enhanced green fluorescent protein fusion encapsulated in PEG-PLA. The designed PEG–PLA microparticles can effectively deliver proteins to the outer segment of the retina, without any apparent cytotoxic effects.

Kirby et al. (Kirby *et al.*, 2013) examined the potential of nanoparticles of poly(lactic-co-glycolic acid) (PLGA) polymers with increasing degree of pegylation (PLGA-PEG) in deliver loperamide to the brain of a mouse. Nanoparticles were prepared dissolving the polymer in ethyl acetate and coumarin-6 dye or loperamide. In vitro and in vivo studies were performed, it was demonstrated that nasal route for drug administration offers a potential noninvasive route for the delivery and targeting of therapeutic agents to the brain. The nasally delivered loperamide is rapidly cleared from the brain, whereas the triblock PLGA PEG loperamide produces a prolonged analgesic effect.

1.2.3.3 Processes and packaging

PLA has been used in various forming processes, such as extrusion molding, injection molding, blow molding, extrusion foaming, fibers and nonwoven fabric, monofilament yarn (Obuchi and Ogawa, 2010). It has a special interest as a matrix in composite materials and a growing interest has been shown, in the last decade, for fully biobased and biodegradable composites based on natural fibres. Some products of natural fibres reinforced PLA have applications on automotive, mobile phone or plant pots (Graupner *et al.*, 2009; Sujaritjun *et al.*, 2013; Le Moigne *et al.*, 2014). Le Moigne et al. (Le Moigne *et al.*, 2014) studied PLA biocomposite with flax fibres for injection moulding applications. The biocomposite was treated with organosilane in order to increase the hydrophobicity and improve the stiffness, the yield stress and the impact strength, while reducing largely the scattering.

Studies have been carried out with plasticizers in order to enhance low melt strength, a problem when blow molding or film extrusion are used as processing methods. However, additives bio-based and biodegradable should be used to maintain PLA composite as green as possible (Herrera *et al.*, 2015). Herrera et al. (Herrera *et al.*, 2015) prepared with a co-rotating twin-screw extruder, a nanocomposites of PLA-cellulose nanofibers (CNF) and glycerol

triacetate as plasticicizer. PLA Plasticized increased degree of crystallinity, improved elongation at break and work of fracture and decreased optical transparency. Further addition of CNF resulted in a plasticized PLA–CNF nanocomposite with improved properties as the elongation at break and the work of fracture.

The use of biopolymers in packaging, especially in food, is increasing due to biodegradability of material because plastic packaging materials are contaminated by foodstuff and biological substance, which makes the recycling of these materials impracticable and most of the time not convenient economically (Siracusa *et al.*, 2012). A packaging material for use in food, due to the contact with food, must be recognized as safe material. Food and Drug Administration (FDA) recognize PLA as safe. The migration of the LA from packaging to food is observed, however, studies have shown that the level of LA that migrates is much lower than the amount of LA used in common food ingredients (Conn *et al.*, 1995). For food packaging, the use of PLA is limited because of the ductility, thermal and barrier properties (Arrieta *et al.*, 2013; Martino *et al.*, 2011). For enhancing the properties, the use of substances has been studied. These substances must be approved as safe for food application. The effect of the addition of limonene in PLA was investigated by Arrieta et al. (Arrieta *et al.*, 2013). Limonene can be found in different citrus and it is one of the most important contributors to citrus flavor. The diffusion of limonene has been widely studied in different materials. The limonene incorporated in PLA films was a good plasticizer, increased the elongation at break and decreased the elastic modulus. As a result of the increase of the chain mobility, the barrier properties were reduced. Recent studies have been conducted to use substances to improve PLA characteristics for food packaging and the use of antimicrobians to prevent food contamination by microorganisms. Researches using antimicrobial substance incorporated into PLA films are limited. The substances that were studied in PLA films are silver (Martínez-Abad *et al.*, 2014), propolis (Mascheroni *et al.*, 2010), olive leaf extract (Özge Erdohan *et al.*, 2013), natamycin (Lantano *et al.*, 2014), nisin (Jin *et al.*, 2009), lemon extract, thymol and lysozyme (Del Nobile *et al.*, 2009).

1.2.4 PLA global market

The demand for bioplastics is continuously rising, and the market is characterized by high and steady growth rates of between 20-100 percent per year. The global production capacity in 2013 for bioplastics amounted to 1.6 million tonnes. Partially biobased PET is leading the field, which was accounting for approximately 40 percent of the global bioplastics

production capacity in 2013. Furthermore, by the year 2018, the production of bioplastics is projected to increase to over 6.7 million tonnes and the main contributors will be PLA and PHA. PLA production represents 11.4% (Aeschelmann and Carus, 2015). Worldwide annual PLA production capacity is expected to be as high as 216,000 metric tons in 2015 (Mazzoli *et al.*, 2014).

DuPont patented a high-molecular weight PLA, in 1954, and then many companies have come forward to commercialize PLA (Madhavan Nampoothiri *et al.*, 2010). Nature Works LLC produce biopolymers Ingeo for applications in packaging, fibers and textiles. Corbion Purac developed lactides and biopolymers for packaging, consumer, fibers, non-woven and automotives. For medical applications, Purasorb® has been commercially for drug systems delivery and medical devices. Some commercially available products are LACEA (Mitsui Toatsu, Japan), LACTY (Shimadzu, Japan), SOLANYL (Rodenburg Biopolymers, Holland), LACTRON (Kanebo Goshen, Japan), GALACTIC (Belgium), PHUSILINE, SYSORB, BIOFIX and PL-FIX (Madhavan Nampoothiri *et al.*, 2010).

1.3 PERSPECTIVES

In recent years, the lactic acid demand has increased that was pushed mainly by the tendency for use of biodegradable plastic such as PLA. In this way, new research has emerged in order to improve productivity and lower production costs of lactic acid. New researches are turned to the genetic improvement of strains, use of lignocellulosic wastes as alternative substrates and development of new model of bioreactors including membrane reactor systems.

Significant advances on lactic acid production have been achieved in the development of fermentation processes in order to solve the cost-sustainable of the biorefineries. The optimization of fermentation processes and alternative fermentation strategies, such as the use of cheap and abundant biomass, starch and lignocellulose. Other potential tools are metabolic pathways engineering for the use of complex substrates without the need of exogenous addition of saccharification pre-treatments.

The capacity of biopolymers production is increasing over the years, however, PLA is not a new polymer by the reason that it has been developed for many years, by several companies around the World. The application of PLA is limited due to its high cost and its limited properties. The demand for improvement of PLA properties is increasing and researches use several materials for this purpose. The use of natural fibers as reinforcements with PLA is of significant interest, since it can lead to the development of a new range of low-cost

biodegradable composites with tailored properties. Some challenges need to be overcome specially the compatibility between hydrophobic PLA and hydrophilic natural fibers. The hydrophobicity is a problem for biomedical application. Also, the use of products, which is considered GRAS to enhance PLA propertie. It, is important for food packaging due to its contact with the food.

The polymerization process using lactide, ring opening polymerization, is the method that carried out high molecular weight of polymer but the time required for the reaction is still very long. Alternatives for the reaction as the use of microwave heating process can obtain similar results at lower temperatures, less time required, lower product oxidation, with high stereopurity and lower energetic costs.

CHAPTER 2 – LACTIC ACID PRODUCTION BY FERMENTATION USING POTATO PROCESSING WASTE AND SUGAR CANE JUICE AS SUBSTRATE

ABSTRACT

Lactic acid (LA), an important molecule applied in the food, pharmaceutical and chemical industry, mainly in the production of biodegradable polymers. Industrially, it has been produced by fermentation. Microorganisms such as bacteria, yeast and fungi produce lactic acid. Lactic acid bacteria (LAB) are a group of related bacteria, including *Lactobacillus*, that produce lactic acid as major metabolic product from carbohydrates by fermentation. Nutritional requirements of bacteria increases the cost of production of lactic acid so alternatives substrates have been studied in order to reduce the cost of production. The fermentation process was developed using potato processing waste and sugarcane juice because of their high carbon source concentration. Potato processing waste was submitted to hydrolysis in order to convert starch to glucose. LA production by fermentation was optimized using, statistical experimental design approach steps of optimization involved the screening of bacteria of the genus *Lactobacillus* and definition of medium composition Kinetics studies in Erlenmeyer flask and stirred tank reactor were also carried out. LA production reached 150 g/l using potato processing waste, it was and 225 g/l with sugar cane juice after 96 hours of fermentation. The use of baker's yeast as a source of nitrogen and nonsterile conditions demonstrated good alternatives for an industrial production process of LA.

KEYWORDS: lactic acid; *Lactobacillus pentosus*, potato processing waste; sugar cane juice

2.1 INTRODUCTION

Lactic acid (LA) is the popular name given to hydroxypropanoic acid ($C_3H_6O_3$). The molecule has an asymmetric carbon, it exists in the form of two optically active isomers: the L(+)lactic acid or S-lactic acid and D(-)lactic acid or R-lactic acid. LA has a large variety of applications primarily in the food industry, but is also applied in the pharmaceutical and chemical industry, with a strong focus on the production of biodegradable polymers (Datta *et al.*, 1995; Wee *et al.*, 2006; Gao *et al.*, 2011).

Production of LA may be carried out by chemical synthesis and by fermentation. When chemically synthesized, LA consists of a racemic mixture (50/50) of D and L forms. LA obtained by fermentation is optically active (L, D or DL), depending on the producing microbial strain, resulting in significant different properties when polymerized (Narayanan *et al.*, 2004). Bacteria and fungi have been reported as microorganisms that produce LA. The LA bacteria

consists of a group of bacteria, which produces LA as a major metabolic product (Reddy *et al.*, 2008).

Carbon sources used for LA production can be sugars such as glucose, sucrose, lactose, etc. These carbon sources have a higher cost, so the use of substrates containing carbon sources such as cassava bagasse, sugar cane bagasse, molasses, whey, starches, among others can reduce the cost of LA production (Madhavan Nampoothiri *et al.*, 2010).

Potato (*Solanum tuberosum L.)* is a tuber of great importance on the nutritional aspect, because it has lots of carbohydrates, and other components such as protein, vitamins, fiber, minerals, potassium and low amount of lipids. The production in Brazil at year of 2014 was 3,689,836 tons (Marques, 2016). It is usually consumed fresh, but its industrial products such as starch and potato chips are of great importance (Carvalho *et al.*, 2014). The fried potato production process, such as straw potato and potato chips generates a residue with high starch content. The peeled potato is cut according to the final product after cutting and the slices must be washed with water to remove the liberated starch in the potato surface, thus generating the by-product of potato processing waste (Vendruscolo and Zorzella, 2002). In Brazil, 200,000-250,000 tons/year of frozen pre-fried potato and 250,000-300,000 tons of chips potato are produced (Shimoyama, 2014). During potato chips manufacturing, much starch is released, up to 1% by weight of the raw material. Potato starch could be recovered by filtration because it causes major environmental problems. Potato starch is generally used for feed (Gélinas and Barrette, 2007).

Sugar cane juice is a very nutritious beverage consumed in Brazil. The broth is extracted from the sugar cane milling process. It is a viscous liquid, color ranging from brown to dark green (due to the presence of chlorophyll and phenolic compounds), basically consisting of water (80%) and total dissolved solids (20%). Of the total solids are sugars include sucrose (17%), glucose (0.4%), and fructose (0.2%), nitrogenous substances, fats, pectins, organic acids and ash. The composition varies according to the variety, age and health of sugar cane, soil, climate and agricultural planning (Cheavegatti-Gianotto *et al.*, 2011; Oliveira *et al.*, 2007).

Due to the high carbon source concentration both potato processing waste and sugar cane juice are good substrates for the production of lactic acid by fermentation. Therefore the goal of this work was to develop step the lactic acid production process is submerged fermentation and bacteria of *Lactobacillus* genus.

2.2 MATERIAL AND METHODS

2.2.1 Strain maintenance

Strain *Lactobacillus agilis* NRRL 14856, *Lactobacillus pentosus* NRRL B-227, *Lactobacillus amylophillus* NRRL B-4437 and *Lactobacillus sp.* LPB-7 were cultivated in MRS (Man–Rogosa–Sharpe) broth at 35ºC during 24 hours. After incubation, glycerol was added to the culture and the broth was stored at -20 ºC and periodically renovated.

2.2.2 Substrate

Potato processing waste (PPW) was obtained from Wanflo Industry (Campo Largo, Brazil). PPW was submitted to acid hydrolysis using hydrochloric acid at 1.5% (w/w) concentration at 121 ºC and 15 minutes according to Woiciechowski et al. (2002). After hydrolysis, the pH was adjusted to 7.0. The hydrolyzed potato processing waste, called HPPW, was used in LA production.

Sugarcane juice (SJ), juice extracted from sugarcane, was submitted to a filtration process in order to remove solids. The juice was also employed in LA synthesis.

2.2.3 Lactic acid production using potato processing waste as substrate

2.2.3.1 Screening of microorganisms for lactic acid production using HPPW and yeast extract

The screening of microorganisms for LA production was conducted using 4 strains of *Lactobacillus* (*L. agilis* NRRL 14856, *L. pentosus* NRRL B-227, *L. amylophillus* NRRL B-4437 e *Lactobacillus spp.* LPB-7). The inoculum from each strain was propagated in MRS broth for 24 hours and then, they were utilized at 10% (v/v) of inoculum rate at production medium composed by 50 g/l of total reducing sugar, 10 g/l of yeast extract and 40 g/l of $CaCO_3$.

The fermentation was carried out in 125 ml Erlenmeyer flask containing 75 ml of culture medium, at 30ºC, 120 rpm for 72 hours. Samples were collected for analysis of LA and sugars (glucose and maltose) concentration by high performance liquid chromatography (HPLC).

2.2.3.2 Inoculum preparation

In order to replace MRS broth for *L. pentosu's* growth of, a culture medium composed by HPPW (20 g/l of total sugars) and yeast extract (YE) was studied at different concentrations (10, 15, 20 and 25 g/l). The preparation of inoculum occurred in triplicate in 125 ml Erlenmeyer

flasks containing 75 ml of culture medium with 10% (v/v) of inoculation rate at 30ºC, 120 rpm during 24 hours and after that, it was analyzed viable cells by plate counting method and total biomass by dry weight method.

A kinetics study of inoculum growth was then conducted in 125 ml Erlenmeyer flasks containing 75 ml culture medium (20 g/l of total sugars and 25 g/l of yeast extract), with 10% (v/v) of inoculation rate in the following growth conditions: 30ºC, 120 rpm till 30 hours. Samples were withdrawn every 6 hours. Total biomass by dry weight method and viable cells were determined by plate counting method.

2.2.3.3 Optimization of lactic acid production

The optimization of LA production was carried out using the experimental designs stretegy. In the first step, the influence of nitrogen source on LA production was conducted. A screening of different nitrogen sources was carried out using imported yeast extract (HiMedia), Brazilian yeast extract (Biorigin YE-MF and Biorigin YE-CMF), peptone, urea, $(NH_4)_2SO_4$ and $(NH_4)_2HPO_4$ (Table 2-1). Each nitrogen source was tested individually in culture medium composed by 50 g/l of total reducing sugars from HPPW and 40 g/l of $CaCO_3$.

Table 2-1 Different nitrogen sources for lactic acid production

Nitrogen Source	Concentration (g/l)
Control	N
YE (HiMedia)	10.0
YE (Biorigin YE-MF)	10.0
YE (Biorigin YE-CMF)	10.0
Peptone	10.0
Urea	2.4
$(NH_4)_2SO_4$	5.2
$(NH_4)_2HPO_4$	5.2

N – not present.

After the study of the influence of nitrogen source, a Plackett & Burman (PB) experimental design was used to test the influence of previous screened YE (10 g/l) and other components present in MRS broth such as K_2HPO_4 (2 g/l), sodium acetate (5 g/l), Na_2SO_4 (2 g/l), $FeSO_4$ (0.05 g/l), $MnSO_4$ (0.05 g/l), tween-80 (1 g/l) and $CaCO_3$ (40 g/l), on the LA production in 50 g/l of reducing sugar from HPPW according to the matrix generated for experimental design (Table 2-2).

Table 2-2 Plackett & Burman (PB) experimental design for screening of nutrients for lactic acid production using HPPW as substrate

Variable	Level	
	-1	+1
Yeast Extract (g/l)	0	10.00
K_2HPO_4 (g/l)	0	2.00
Sodium Acetate (g/l)	0	5.00
Na_2SO_4 (g/l)	0	2.00
$FeSO_4$ (g/l)	0	0.05
$MnSO_4$ (g/l)	0	0.05
Tween 80 (g/l)	0	1.00
$CaCO_3$ (g/l)	0	40.00

The last step of optimization was then determining the concentration of total reducing sugars, yeast extract and $CaCO_3$ concentration using an experimental central composite rotatable designs (CCDR) 2^3 totalizing 17 essays (Table 2-3).

Table 2-3 Central composite rotatable designs (CCDR) experimental design for optimization of LA production using HPPW as substrate

Variable	Level				
	-1,68	-1	0	+1	+1,68
Reducing sugars (g/l)	50	70	100	130	150
Yeast extract (g/l)	1,6	5	10	15	18
$CaCO_3$ (g/l)	46	60	80	100	114

All the fermentation steps were carried out in a 125 ml Erlenmeyer flask containing 75 ml of culture medium, 10% (v/v) of inoculum rate at 30ºC, 120 rpm for 72 hours. After fermentation, lactic acid concentration was analyzed by HPLC.

2.2.3.4 Kinetics of lactic acid production using HPPW in Erlenmeyer flask and Stirred Tank Reactor (STR)

LA production using HPPW was carried out in Erlenmeyer flasks and in a 7 l stirred tank reactor (STR) (Laboratory fermentor model MDL, B.E. Marubishi, Thailand). In both systems, the medium was composed by 160 g/l of reducing sugars, 10 g/l of yeast extract and 80 g/l of $CaCO_3$. The inoculum rate was 10% (v/v) previously grown in medium 20 g/l of total sugar and 25 g/l of YE. In 125 ml Erlenmeyer flasks, with 75 ml of medium were incubated at 30ºC and 120 rpm during 96 hours. In a 7 l STR, 4 l of medium were incubated at 30ºC, 150 rpm during 96 hours. Samples were withdrawn periodically for lactic acid analysis and sugars.

2.2.4 Lactic acid production using sugarcane juice (SCJ) as substrate

2.2.4.1 Inoculum preparation

The strain of *L. pentosus* stored in glycerol at -20°C was reactivated in MRS broth at 35°C for 24 h. After growth, the culture was used to inoculate sugarcane juice (SCJ) in fermentation studies .

The growth of *L. pentosus* inoculum was studied in medium composed by SCJ and yeast extract in order to replace the MRS broth. The culture medium consisted of 20 g/l of total reducing sugars and yeast extract (20 and 25 g/l). A control medium was tested using MRS broth.

The propagation of inoculum occurred in triplicate in 125 ml Erlenmeyer flask with 75 ml of culture medium with 10% (v/v) of inoculation rate at 30ºC and 120 rpm during 24 hours. Viable cells and total biomass were then quantified by plate counting method and dry weight method respectively.

2.2.4.2 Optimization of lactic acid production in sugarcane juice substrate

The first step of optimization of LA production included the study of the influence of nitrogen sources Yeast extract, peptone, urea, amonium sulphate and monobasic amonium phosphate were tested. The medium was composed by SCJ 100 g/l (total sugar), 55 g/l of $CaCO_3$ and nitrogen source. The concentrations of nitrogen sources were standardized in terms of the amount of total nitrogen concentration of 1 g/l in the culture medium. Triplicates for each source and a control were analyzed.

The next step of optimization aimed to study the nutritional conditions of LA production of varying the concentrations of total reducing sugars, YE and $CaCO_3$. The optimization was based on an experimental design central composite rotational design (CCRD) 2^3, 8 runs, 6 runs in the axial points and 3 runs in the central point totalizing 17 experiments (Table 2-4).

Table 2-4 CCDR experimental design for optimization of LA production

Variable	Level				
	-1,68	-1	0	+1	+1,68
Total sugar (g/l)	66	100	150	200	234
Yeast extract (g/l)	6.6	10	15	20	23.4
$CaCO_3$ (g/l)	56	70	90	110	124

LA production by fermentation was performed in 125 ml Erlenmeyer flasks containing 75 ml culture medium with 10% (v/v) of inoculation rate, stirring at 120 rpm and temperature 30°C for 72 hours.

2.2.4.3 Kinetics of lactic acid production using sugar cane juice in Erlenmeyer flasks and stirred tank reactor

LA production using SJC was carried out in Erlenmeyer flasks and in a 7 l stirred tank reactor (STR) (Laboratory Fermentor Model MDL, B.E. Marubishi, Thailand). The medium was composed by optimized conditions: 230 g/l of reducing sugars, 15 g/l of yeast extract and 90 g/l of $CaCO_3$. The inoculum rate was 10% (v/v). In 125 ml Erlenmeyer flasks with75 ml of culture medium were incubated at 30ºC, 120 rpm during 96 hours. Fermentation was also carried out in a 7 l STR, with 4 l of medium at 30ºC, 150 rpm during 48 hours.

2.2.5 Production of lactic acid using baker's yeast as nitrogen source

The aim of using baker's yeast (BY) in the culture medium was to replace the nitrogen source (yeast extract) and, thus, reduce the production cost of LA. In this way, a comparative experiment using different nitrogen sources was conducted for LA production. Previously, optimized medium, based on potato processing waste and sugarcane juice were used for LA production. Fermentation was carried out in 125 mL Erlenmeyer flasks with 75 ml of culture medium with 10% (v/v) of inoculation rate at 30°C at 120 rpm for 72 hours.

2.2.6 Lactic acid production in non-sterile conditions

A different strategy of non-sterile conditions for fermentation was developed aiming to decrease LA production costs of sterilization process. This study was carried out in 125 mL Erlenmeyer flasks containing 75 ml of non-sterile culture medium (150 g/l of total sugars from HPPW, 10 g/l of YE and 80 g/l of $CaCO_3$) with 10% (v/v) of inoculation rate at 30°C under stirring at 120 rpm for 72 hours.

2.2.7 Analytical methods

Fermented broth was acidified with sulphuric acid to convert calcium lactate to LA. After that, it was centrifuged at 1800 x g (times gravity) for 20 minutes (Centribio, model 80-2B). The supernatant was filtered through a 0.22 µm cellulose acetate membrane.

LA and reducing sugars content was analyzed by high performance liquid chromatography (HPLC) (Shimadzu LC 10AD, detector RID 10A), using a Aminex HPX-87

H column, mobile phase of 5mM H_2SO_4, 0.6 mL/min, at 60ºC. Standards for chromatography analyses were L-lactic acid 99% (Sigma Aldrich), D-glucose (Sigma Aldrich) and D-maltose (Sigma Aldrich).

Total biomass was determined by dry weight method viable cells were determined by plate counting method (Grigorova and Norris, 1990).

The concentration of the isomers D and L were determined by D-Lactate Colorimetric Assay Kit (Sigma-Aldrich, USA). In this assay, D-Lactate is specifically oxidized by D-Lactate hydrogenase and generates a proportional colorimetric product measured at 450 nm. The reagents were added according to Table 2-5, incubated the reaction for 30 minutes at room temperature and measured the absorbance at 450 nm.

Table 2-5 Reaction Mixes for D-lactate determination

Reagent	Sample Blank	Samples and Standards
D-Lactate Assay Buffer	48 mL	46 mL
D-Lactate Enzyme Mix	–	2 mL
D-Lactate Substrate	2 mL	2 mL

2.2.8 Statistical analysis

Results of experimental designs were analyzed by the Software Statistica 5.0 (StatSoft, Tulsa, USA).

2.3 RESULTS AND DISCUSSION

2.3.1 Production of lactic acid using potato processing waste as substrate

2.3.1.1 Screening of strains for lactic acid production

The screening of strains with greater LA production capacity was conducted using hydrolyzed potato processing waste (HPPW) as substrate. All studied strains (*L. agilis* NRRL 14856, *L. pentosus* NRRL B-227, *L. amylophilus* NRRL B-4437 and *Lactobacillus sp.* LPB-07) produced a great amount of LA, which means more than 40 g/l with a yield above 84% (Table 2-6), before optimization of fermentation conditions. The HPPW contains high amount of glucose and maltose in less quantity. These sugars are easily metabolized by microorganism allowing the production of LA. The strain that produced the highest amount of LA was *L.*

pentosus NRRL B-227, with 47.2 g/l of LA representing a productivity of 0.65 g/l.h. Therefore, the following studies of LA production were performed with this strain.

Table 2-6 Screening of strains for LA production in hydrolyzed potato processing waste

Strain	Lactic acid (g/l)	Residual Sugar (g/l)	Yield (%)	Productivity (g/l.h)
L. agilis NRRL 14856	42.3 ± 2.3	5.3 ± 0.3	94	0.58
L. amylophilus NRRL B-4437	40.7 ± 1.5	1.7 ± 0.2	84	0.56
L. pentosus NRRL B-227	47.2 ± 0.1	2.7 ± 0.2	99	0.65
Lactobacillus sp. LPB-07	43.1 ± 1.2	1.6 ± 0.2	89	0.60

2.3.1.2 Inoculum preparation

After the screening of strains, *L. pentosus* NRRL B-227 growth was evaluated in a medium composed by HPPW and YE. This study aimed to replace the MRS broth used in the propagation of the strain, which could lead to a reduction in the LA production costs.

First, the growth of *L. pentosus* NRRL B-227 was studied in MRS broth and a culture media based in 20 g/l of total sugars and different concentrations of yeast extract - 10, 15, 20 and 25 g/l. The results showed an increase in biomass with the increase of nitrogen source concentration (Table 2-7). This fact shows that a more equilibrated C/N ratio is required for *L. pentosus* growth. The majority of lactic acid bacteria (LAB) require nutrients such as amino acids, peptides, nucleotides and vitamins for growth and production of LA because of its limited biosynthesis capacity (Lahtinen *et al.*, 2011; Hofvendahl and Hahn–Hägerdal, 2000; Abdel-Rahman *et al.*, 2013).

Biomass production was similar in MRS broth and the culture medium formulated with 25 g/l of YE reaching 3.11 g/l of dry biomass of viable cells, and $5{,}0x10^{11}$ colonies forming units (CFU)/ml and 2.70 g/l and $4{,}9x10^{11}$ CFU/ml, respectively.

Table 2-7 Resutls of growth of L. pentosus in formulated medium containing hydrolyzed potato processing waste and yeast extract

Run	Condition	Biomass (g/l)	Viable cells (CFU/ml)
1	20 g/l of total sugar and 10 g/l of YE	1.41 ± 0.14	$5.0x10^{09} \pm 4.2x10^{08}$
2	20 g/l of total sugar and 15 g/l of YE	1.36 ± 0.29	$1.0x10^{10} \pm 1.0x10^{08}$
3	20 g/l of total sugar and 20 g/l of YE	1.71 ± 0.30	$1.2x10^{11} \pm 4.2x10^{09}$
4	20 g/l of total sugar and 25 g/l of YE	2.70 ± 0.27	$4.9x10^{11} \pm 7.1x10^{09}$
5	MRS broth	3.11 ± 0.30	$5.0x10^{11} \pm 5.7x10^{09}$

Analysis of variance (ANOVA) compares three or more treatments. In this study, different conditions correspond to the treatments. The ANOVA table for the results of biomass concentration (Table 2-8) demonstrates for 5% of significance level, p-value is 0.004, which means different conditions of culture medium influenced significantly on biomass concentration.

Table 2-8 One way ANOVA for inoculum preparation

	Degrees of freedom	Sum of Squares	Mean Square	F	P-value
Factor	4	5.073	1.268	17.628	0.004
Residue	10	0.360	0.072		

According to the Tukey test (Table 2-9), which analyzes the equality of values at 5% level of significance, the results of biomass concentration using MRS medium and culture medium composed by 20 g/l of total sugar from HPPW and 25 g/l of YE are similar (p = 0.588). Comparing others cultures medium to MRS, p-value obtained is lower than 0.05. In the culture medium formulated with 20 g/l of total sugars and 25 g/l of YE, the concentration of sugars and nitrogen corresponds to the same that is present in MRS broth. Results indicate that it is possible to replace the MRS medium by the optimized medium containing 20 g/l of total sugar and 25 g/l of YE.

Table 2-9 Results of Tukey test for inoculum preparation

Level	P-value
10 g/l of YE – 15 g/l of YE	1.000
10 g/l of YE – 20 g/l of YE	0.786
10 g/l of YE – 25 g/l of YE	0.025
10 g/l of YE – MRS broth	0.008
15 g/l of YE – 20 g/l of YE	0.687
15 g/l of YE – 25 g/l of YE	0.021
15 g/l of YE – MRS broth	0.007
20 g/l of YE – 25 g/l of YE	0.070
20 g/l of YE – MRS broth	0.018
25 g/l of YE – MRS broth	0.588

The kinetics of *L. pentosus* NRRL B-227 growth in MRS broth and HPPW/YE medium containing 20 g/l of total sugar and 25 g/l of YE was performed. The lag phase (adaptation) is very short, less than 6 hours, in both culture media (Table 2-10) indicates that both culture media, MRS broth and,. The exponential growth phase lasts till 24 hours when biomass reached

3.16 g/l in MRS broth and 2.54 g/l in HPPW/YE medium. After that, the stationary phase started.

Maximum growth rate was obtained after 6 hours and the growth rate increased until 18 hours whith MRS medium. For HPPW/YE medium, the growth rate decreased after 6 hours. In this way, the inoculum was then cultivated during at least 18 hours with HPPW/YE medium.

Table 2-10 Kinetics *L. pentosus* NRRL B-227 growth in MRS broth and HPPW/YE medium

	MRS broth		HPPW/YE	
Time	Biomass concentration (g/l)	Growth Rate (h^{-1})	Biomass concentration (g/l)	Growth Rate (h^{-1})
0	0.729±0.010	-	0.714±0.010	-
6	1.543±0.080	0.125	1.357±0.050	0.107
12	2.021±0.080	0.045	1.971±0.120	0.062
18	2.875±0.055	0.059	2.295±0.060	0.025
24	3.157±0.110	0.015	2.536±0.001	0.016
30	3.043±0.100	-0.006	2.450±0.110	-0.005

2.3.1.3 Optimization of lactic acid production

The influence of different sources of nitrogen on LA production by *L. pentosus* NRRL B-227 is shown in Figure 2-1. The yeast extracts from different origins, HiMedia Biorigin MF, Biorigin CMF and peptone led to a similar LA production, 47 g/l, 42 g/l, 44 g/l and 43 g/l, respectively. The YE-CMF Biorigin was chosen for the development of the production process, because their advantages over the YE-HiMedia and petone due to its cost that is about ten times lower. Concerning the YE-MF Biorigin brand and YE-CMF Biorigin presented lower hygroscopicity. LA production was higher with complex nitrogen sources such as peptone extract , which contain substances such as amino acids, proteins, vitamins and minerals that are required to lactic acid bacteria. Inorganic nitrogen sources and urea did not promote LA production due to the lack of nutrients (2.3.1.2 Inoculum preparation).

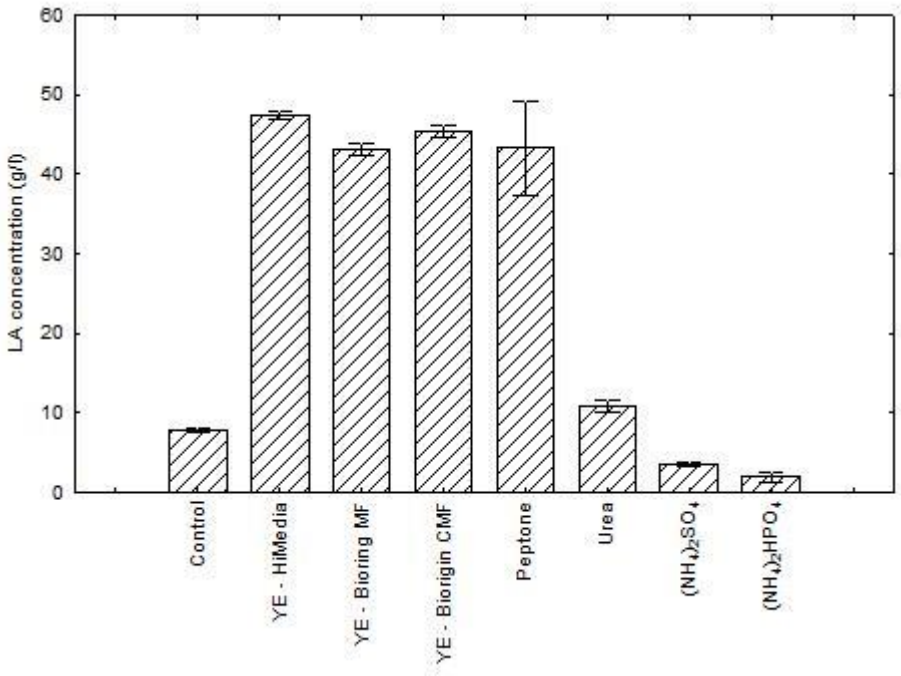

Figure 2-1 Lactic acid production under different nitrogen sources

Nancib et al. (2005) studied LA production using date juice as substrate and different nitrogen sources (yeast extract, ammonium sulfate, tryptic soy, urea, peptone and casein hydrolysate). The medium had the following composition: 50 g/l of glucose (date juice), 0.5 g/l of $MgSO_4x7H_2O$, 0.03g/l of $MnSO_4xH_2O$, 3 g/l of KH_2PO_4, 3 g/l of K_2HPO_4, 2 g/l of sodium acetate, 1 ml/l of Tween 80, different nitrogen sources and B vitamins. Yeast extract showed the greatest enhancing effect on LA production, however authors showed that part of the yeast extract could be replaced by ammonium sulfate and B vitamin.

The selection of nutrients to be added to HPPW was made with the support of Plackett & Burman experimental design. The influence of yeast extract, K_2HPO_4, $C_2H_3O_2Na$, Na_2SO_4, $FeSO_4$, $MnSO_4$, Tween 80 and $CaCO_3$ on LA production was studied (Table 2-11). The highest production of LA was 45.4 g/l (run 1), which contains YE, $C_2H_3O_2Na$, polysorbate 80 and $CaCO_3$. Lower LA yields (4.4 g/l) were obtained in the tests (run 12) without the presence of a nitrogen source (control, without addition of nutrients). This fact, means that a certain C/N is needed for bacteria growth and LA accumulation.

Table 2-11 Screening of medium nutrients for LA production

Run	YE	K_2HPO_4	$C_2H_3O_2Na$	Na_2SO_4	$FeSO_4$	$MnSO_4$	Tween 80	$CaCO_3$	LA (g/L)
1	10	0	5	0	0	0	1	40	45.4
2	10	2	0	2	0	0	0	40	34.8
3	0	2	5	0	0.05	0	0	0	8.4
4	10	0	5	2	0	0.05	0	0	11.3
5	10	2	0	2	0.05	0	1	0	10.1
6	10	2	5	0	0.05	0.05	0	40	42.8
7	0	2	5	2	0	0.05	1	0	8.1
8	0	0	5	2	0.05	0	1	40	9.5
9	0	0	0	2	0.05	0.05	0	40	7.2
10	10	0	0	0	0.05	0.05	1	0	9.7
11	0	2	0	0	0	0.05	1	40	11.5
12	0	0	0	0	0	0	0	0	4.4
13	5	1	2.5	1	0.025	0.025	0.5	20	33.9
14	5	1	2.5	1	0.025	0.025	0.5	20	34.1
15	5	1	2.5	1	0.025	0.025	0.5	20	32.2

The ANOVA of the experimental design was generated with R^2 of 0.734 (Table 2-12). it is possible to verify that YE and $CaCO_3$, are significant for LA production at the studied levels, ($p < 0.05$). Therefore, YE and the $CaCO_3$ were selected as HPPW medium components in the next steps of optimization.

Table 2-12 ANOVA for Placket & Burman experimental design for medium components for LA production (R^2=0.734)

	Sum of Squares	Degrees of freedom	Mean Square	F	p-value
YE	1618.596	1	1618.597	6.732	0.041
KH_2PO_4	123.232	1	123.232	0.512	0.501
$C_2H_3O_2Na$	329.941	1	329.942	1.372	0.286
Na_2SO_4	243.621	1	243.621	1.013	0.353
$FeSO_4$	111.977	1	111.978	0.465	0.520
$MnSO_4$	70.0785	1	70.078	0.291	0.609
Tween 80	34.785	1	34.785	0.144	0.717
$CaCO_3$	1449.559	1	1449.559	6.029	0.049

The third step of LA production optimization included the study of total sugar, YE and $CaCO_3$ concentrations that was performed with the support of a CCDR experimental design 2^3 totalizing 17 essays (Table 2-13). The highest production LA of was 135 g/l using 150 g/l of total sugar, 10 g/l of YE and 80 g/l of $CaCO_3$ (run 10), corresponding to the axial point of total sugar concentration and central point for YE and $CaCO_3$. The lowest LA production was 46.4 g/l, using 50 g/l of total sugar, 10 g/l of YE and 80 g/l of $CaCO_3$ (run 9). With this fermentation

conditions, the C/N ratio is lower compared to the C/N ratio of run10, which does not favor LA production.

Table 2-13 CCDR experimental design for the optimization of total sugar, yeast extract and $CaCO_3$ concentration

Run	Total sugar	Yeast Extract	CaCO3	Lactic acid (g/l)	Yield (%)
1	70	5	60	69.7	99%
2	70	5	100	67.6	97%
3	70	15	60	63.4	91%
4	70	15	100	59.7	85%
5	130	5	60	96.4	74%
6	130	5	100	110.7	85%
7	130	15	60	116.1	89%
8	130	15	100	122.9	95%
9	50	10	80	46.1	93%
10	150	10	80	135.0	90%
11	100	2	80	73.6	74%
12	100	18	80	94.2	94%
13	100	10	46	85.8	86%
14	100	10	114	94.4	94%
15 (C)	100	10	80	97.8	98%
16 (C)	100	10	80	110.4	100%
17 (C)	100	10	80	106.5	100%

The influence of the C/N ratio is significant for LA production according to the Pareto chart (Figure 2-2), which showed the significant interaction between the two variables (p-value < 0.05). Total sugars and YE concentration influenced positively on the LA production as well as the interaction between two variables. $CaCO_3$ concentration did not influence significantly on LA production at concentration range studied, nether the interaction between others variables. The presence of this component in the culture medium is important due to neutralization of lactic acid produced during fermentation, thus forming calcium lactate. One characteristic associated to lactic acid bacteria is the sensibility to change of pH, LA production reduces pH of medium thus inhibiting the lactic acid bacteria (Singh *et al.*, 2006).

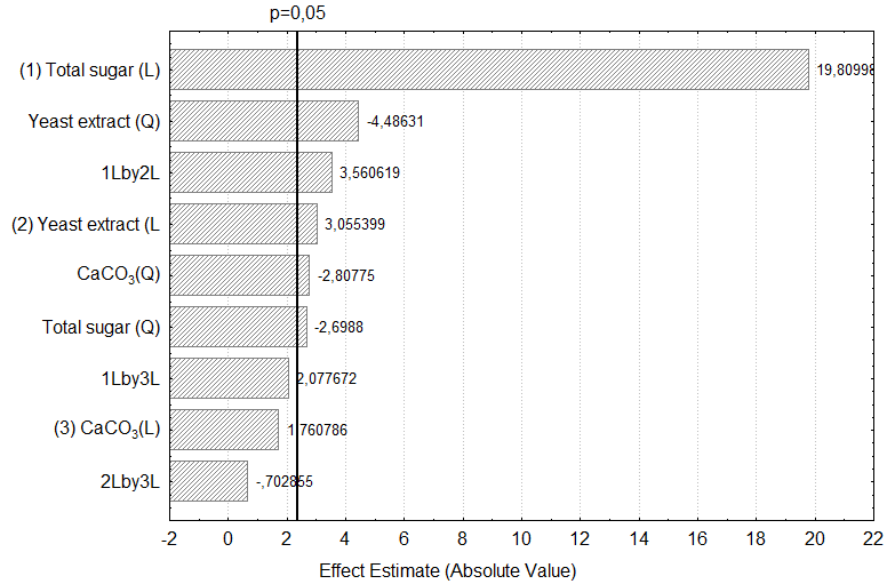

Figure 2-2 Pareto chart for CCDR experimental design (R^2=0,982)

The contour plot was generated (Figure 2-3), which showed the optimum LA production area (dark region). Optimal concentrations of total sugars and YE where observed above 140 g/l and 10 g/l, respectively. The model adjustment of experimental design is 0.98., Although the optimum region is not closed, increasing LA concentrations can be obtained with high total sugar concentrations and central YE concentrations (from 10 to 18 g/L).

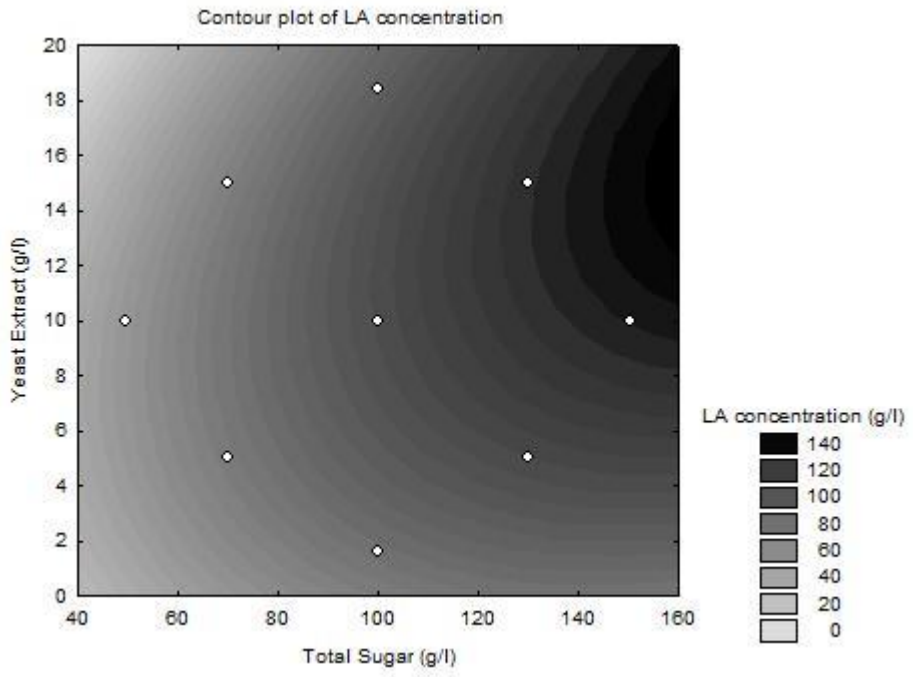

Figure 2-3 Contour plot of HPPW medium composition (total sugar and YE) optimization for LA production

The obtained mathematical model is described by Equation 2-1, where z – LA concentration; x – total sugars concentration; y – YE concentration; w – $CaCO_3$ concentration.

$$z=-49.996+0.813x-0.004x^2+3.117y-0.264y^2+0.0384xy+0.0056wx-0.0114wy+1.156w-0.0093w^2 \quad (2\text{-}1)$$

The results of this experimental design were validated experimentally. According to the equation 2-1, the result of the LA production using 164 g/l of sugar, 10 g/l of YE and 80 g/l of $CaCO_3$ is 140.8 g / l. The experimental result of LA production under these same conditions was 140.1 g/l. After optimization steps, LA production is maximized with 150 g/l of total sugar, 10 g/l of YE and 80 g/l of $CaCO_3$.

2.3.1.4 Kinetics of lactic acid production using hydrolyzed potato processing waste

The kinetics of LA production was studied using HPPW as substrate during 96 hours. LA production and total and reducing sugars consumption can be visualized in Figure 2-4. LA is a primary product of cells' metabolism that is associated with cell growth. LA is produced in the first 12 hours (130.6 g/l) with a high production rate up to 60 hours with. After this period there is an increase in the concentration of LA, but with lower production rate. The highest concentration of LA (150 g/l) was reached 96 hours.

The consumption of sugars occurred with a higher rate in the first 48 hours of fermentation varying from 160 g/l to 28 g/l. At the end of fermentation, residual sugars concentration fell to 13 g/l. The profile of reducing sugars' consumption of was similar to the total sugars' consumption.

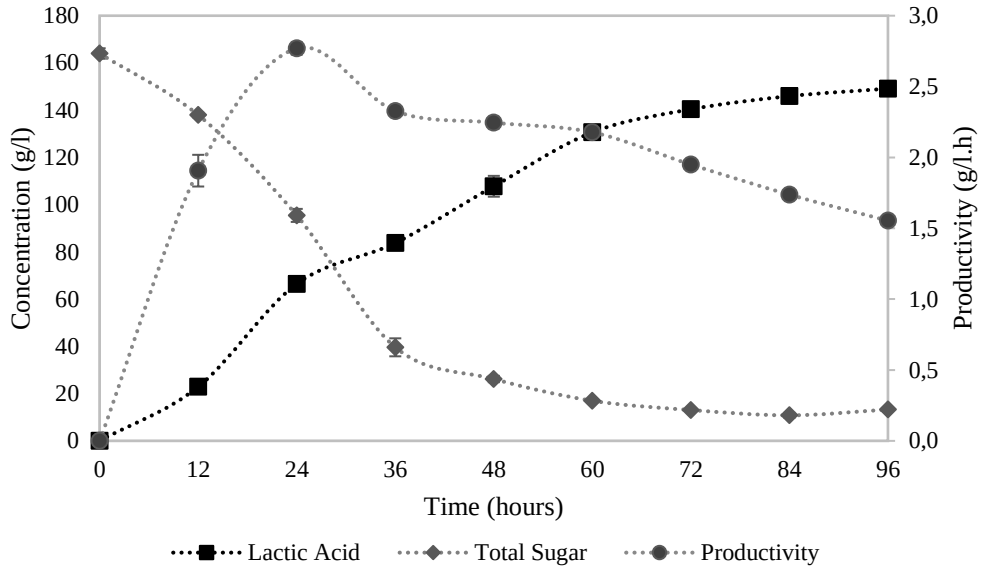

Figure 2-4 Kinect of lactic acid production in HPPW in Erlenmeyer flask

In STR system (Figure 2-5), the maximum LA production was obtained in 48 hours of fermentation. After this time LA and total sugars concentration, which was consumed in the first 48 hours, remained constant. The highest LA concentration reached 117 g/l, obtained from a total sugar concentration of 130 g/l.

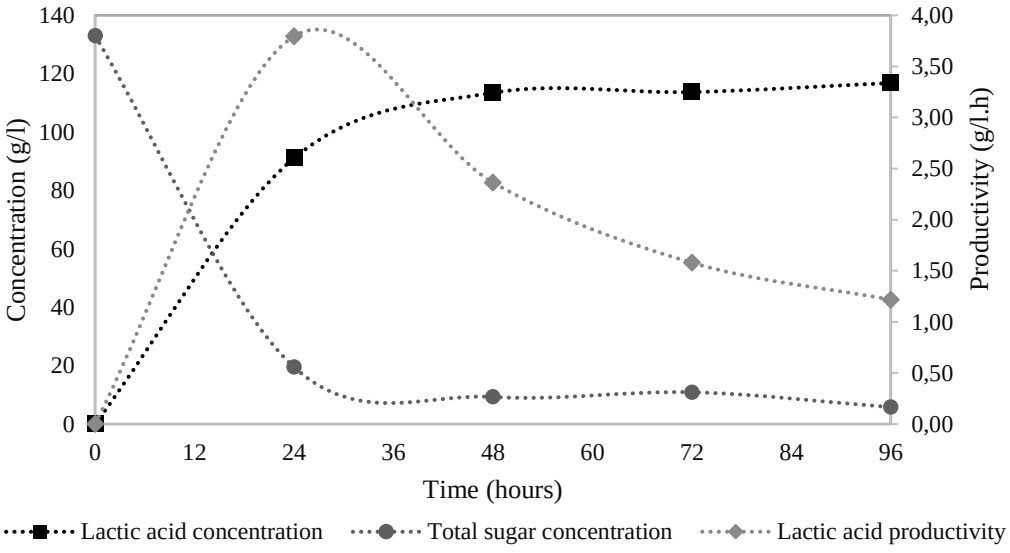

Figure 2-5 Kinect of lactic acid production using HPPW in STR

Comparing the production in different reactors, STR and Erlenmeyer flasks, the scale-up of fermentation from 75 ml (Erlenmeyer flask) to 5 l (SRT) promoted an increase on

productivity of lactic acid, 1.5 g/l.h to 2.4 g/l.h, thus fermentation time was reduced from 96h to 48h because the reactor configuration. The presence of impellers improve agitation of the culture medium and gas-liquid mass transfer of oxygen dissolved in the culture medium.

2.3.2 Lactic acid production using sugarcane juice as substrate

2.3.2.1 Inoculum preparation in sugarcane juice

The results of biomass production using the inoculum prepared with sugar cane juice, with different concentrations of YE, and MRS broth as substrate are shown in Figure 2-6. Biomass production with MRS broth and the culture medium based on sugarcane juice added of 25 g/l of YE presented similar values for dry biomass and viable cells 2.80 g/l and $4.5x10^{11}$ CFU/ml for MRS broth and 2.20 g/l and $4.3x10^{11}$ CFU/ml for sugarcane juice (SCJ) medium containing 25 g/l of YE. These results are similar to those obtained for the HPPW medium with YE. This fact indicate that it is possible to replace MRS medium by the SCJ medium containing 20 g/l of total sugars and 25 g/l of YE as the viable cell values are similar in both cases.

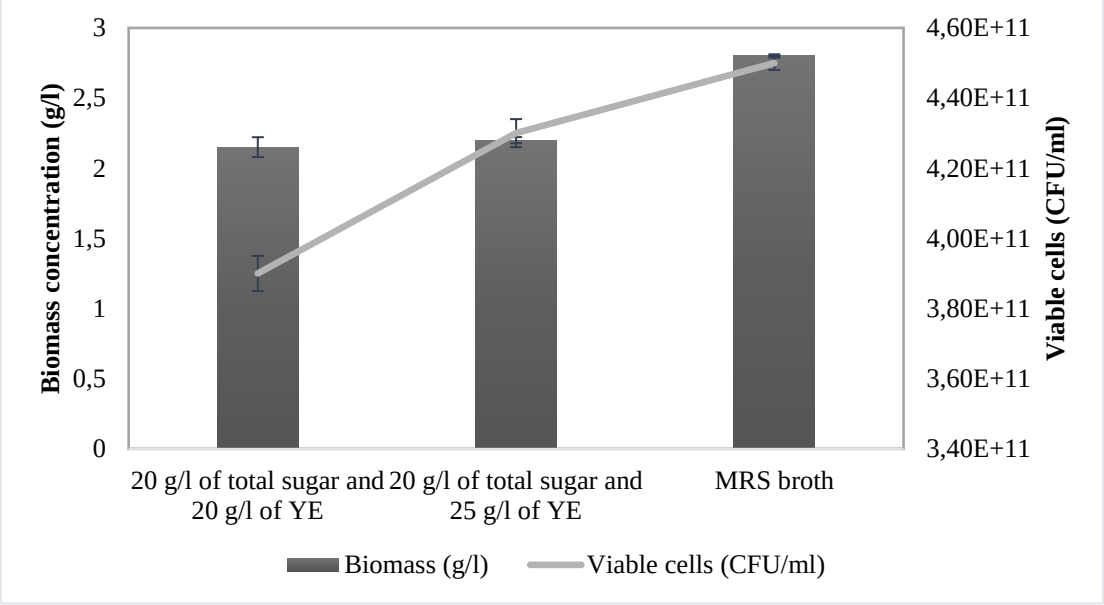

Figure 2-6 Results of growth of *L. pentosus* NRRL B-227 in MRS broth and sugarcane juice based medium with the addition of yeast extract.

2.3.2.2 Optimization of lactic acid production in sugarcane juice as substrate

The effect of different nitrogen sources on LA production with sugarcane juice is shown in Figure 2-7. As in the study with HPPW medium, complex nitrogen sources such as peptone

and YE-CMF Biorigin presented a better effect on LA production. Peptone led to better results in LA production, therefore, due to its lower cost, the YE-CMF Biorigin was selected as the source of nitrogen for further studies.

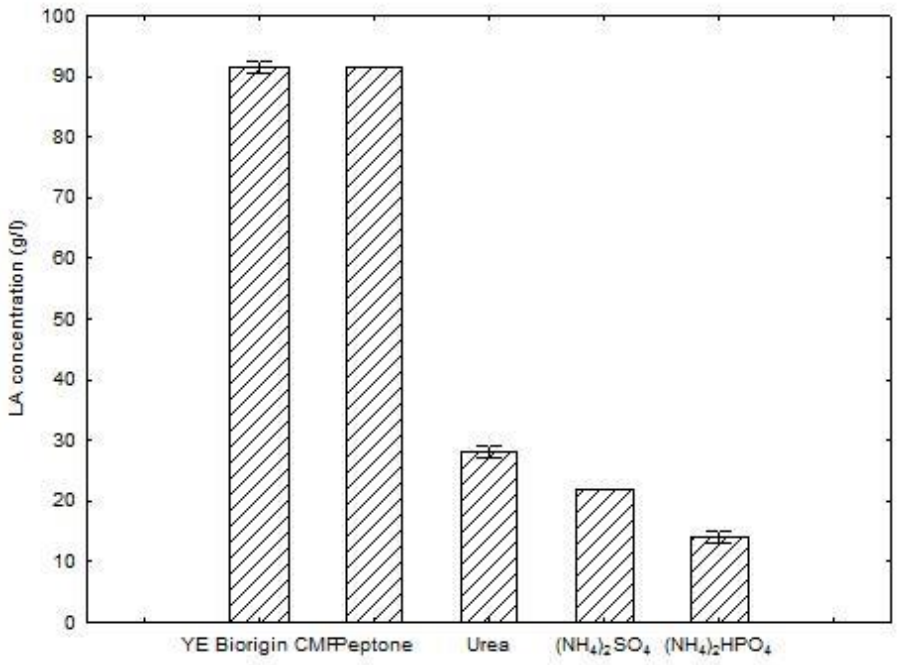

Figure 2-7 LA production in sugar cane juice with different nitrogen sources

As in the study conducted in HPPW, total sugars, YE and $CaCO_3$ concentrations were optimized in SCJ medium. Maximum LA production (199.4 g/l) was obtained under the condition of 234 g/l of total sugars, 15 g/l of YE and 90 g/l of $CaCO_3$, which is the axial point condition for total sugar and central point for the other independent variables (Table 2-14).

In all conditions of fermentation, the yields obtained were above 61%. The highest yield (95%) obtained in condition 7, which does not correspond to greater production of AL (199 g/l 85%) obtained in condition 10. This increase in yield from 85% to 95% corresponds to an increase in the amount of YE culture medium from 15 g/l to 20 g/l, which represents a significant increase in the cost of culture medium because of the cost of YE.

Table 2-14 CCDR experimental design for LA production optimization using sugarcane juice as substrate

Run	Total sugar (g/l)	Yeast extract (g/l)	$CaCO_3$ (g/l)	Lactic acid (g/l)	Yield (%)
1	100	10	70	81.5	82
2	100	10	110	77.7	78
3	100	20	70	74.1	74
4	100	20	110	71.8	72
5	200	10	70	158.5	79
6	200	10	110	156.1	78
7	200	20	70	189.8	95
8	200	20	110	163.5	82
9	65.9	15	90	53.2	81
10	234.1	15	90	199.4	85
11	150	6.6	90	91.1	61
12	150	23.4	90	128.8	86
13	150	15	56.4	122.1	81
14	150	15	123.6	133.0	89
15 (C)	150	15	90	132.1	88
16 (C)	150	15	90	127.9	85

The analysis of interactions between variables by Pareto chart (Figure 2-8), with model adjustment $R^2 = 0.98$, it is possible to observe that in the studied concentration range, only total sugars and YE concentration influenced significantly on LA production ($p < 0.05$). $CaCO_3$ concentration did not influence on LA production however, its presence is important as neutralizant agent.

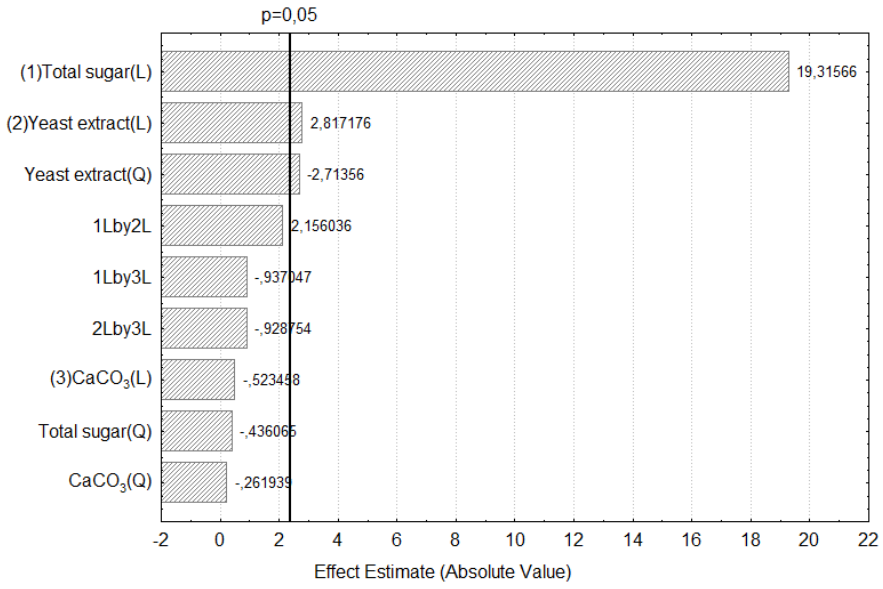

Figure 2-8 Pareto chart of SCJ medium composition (total sugar and YE) optimization for LA production

In the generated contour plot (Figure 2-9) the optimal conditions for LA production includes the region of total sugar concentration above 234 g/l (highest concentration studied). At this concentration, the SCJ was used without dilution. For studies with higher concentrations of total sugar, it would be required a new step for substrate preparation (concentration). Therefore, it was defined following conditions for LA production using SCJ as substrate: 230 g/l of total sugar, 15 g/l of YE and 90 g/l of $CaCO_3$.

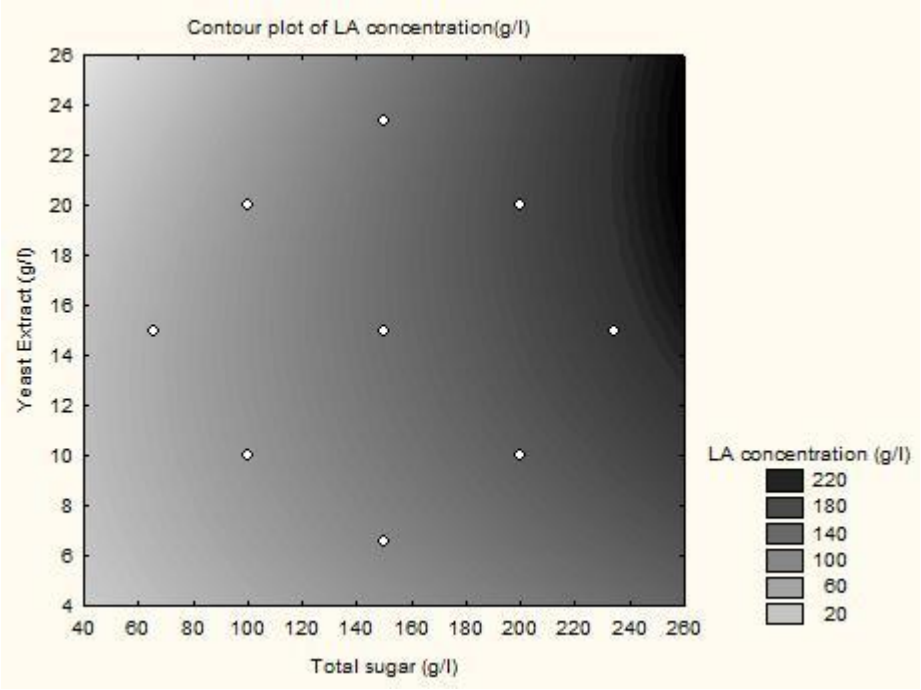

Figure 2-9 Contour plot of optimization concentration of total sugar and YE in SCJ medium for LA production

The mathematical model obtained is described by Equation 2-2, where z–LA concentration; x– total sugar concentration; y – YE concentration and w – $CaCO_3$ concentration.

$$z=-120.843+0.888x-0.0004x^2+8.203y-0.276y^2+0.026xy-0.003wx-0.028wy+0.0003w+0.010w^2 \quad (2\text{-}2)$$

2.3.2.3 Kinetics of lactic acid production using sugarcane juice in Erlenmeyer flask and Stirred tank reactor

The kinetics study of LA production with SCJ as substrate was performed during 96 hours in Erlenmeyer flasks (Figure 2-10). The production of LA was observed at the first hours of fermentation (6.6 g/l in 6 hours).

The maximum LA concentration, 225 g/l, was attained in 96 hours, which means 99% of yield. The productivity increased until 48 hours, 3.6 g/l.h, and then decreased. In the first 12 hours, a low yield of LA production was observed probably due to the bacteria's carbon source consumption for their own growth. After 24 hours the yield increased to 84%.

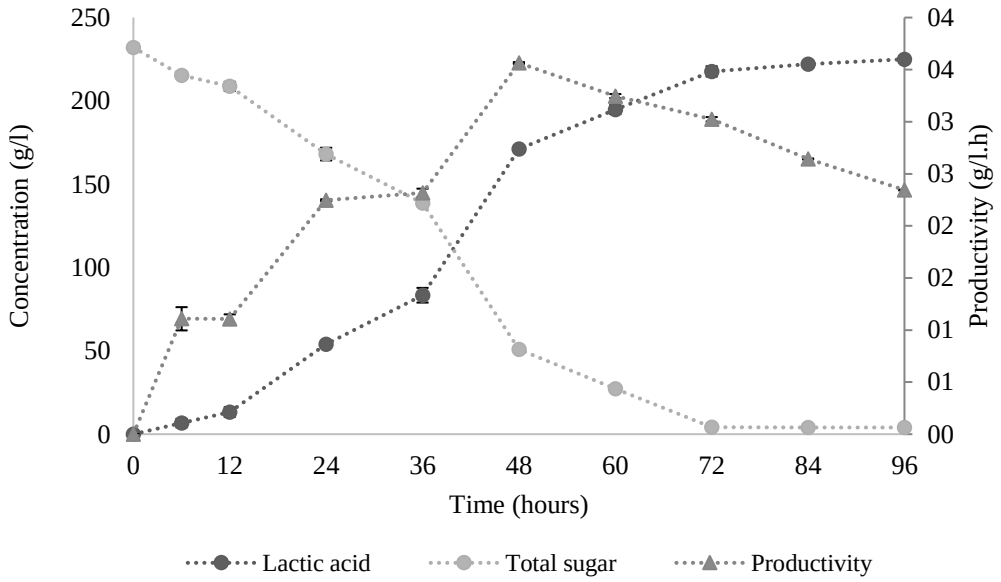

Figure 2-10 kinetics study of LA production and total sugar consumption in Erlenmeyer flasks with sugarcane based medium

Kinetics study of LA production was also carried out in STR bioreactor (Table 2-15). After 48 hours, a solidification of the medium was observed probably due to the high concentration of LA in the form of calcium lactate (Figure 2-11). The solubility of calcium lactate at 30ºC is around 73 g/l (Kubantseva and Hartel, 2002) and at 48 hours of fermentation, calcium lactate concentration in the broth was 211 g/l, which correspond to 176 g/l of LA. The process was then interrupted. Even so, the process yield was 85% and the remaining total sugar concentration in the fermentation broth was 3.9 g/l.

Table 2-15 Kinetics of LA production using SCJ in STR

Time (hours)	Total sugar (g/l)	Lactic acid (g/l)	Productivity (g/l.h)	Yield (%)
0	211	0	-	-
24	104	90	3.8	85
48	4.0	176	3.7	85

Figure 2-11 LA production using SCJ as substrate in STR. Solidification of the medium after 48 hours of fermentation due to the high concentration of calcium lactate

Comparing the LA production in Erlenmeyer flasks and STR, the largest production and yields were obtained in Erlenmeyer flasks, 225 g/l and 99%. The productivity was similar between two reactors, 3.6 g/l.h in Erlenmeyer flask and 3.7 g/l.h in STR.

2.3.3 Production of lactic acid using baker's yeast as nitrogen source

The replacement of yeast extract for baker's yeast as a nitrogen source for production of LA (Table 2-16) showed that the strain *L. pentosus* takes nutrients from baker's yeast for production of LA. In the optimized culture medium based on HPPW, the production of LA was 134.1 g/l using YE and 113 g/l using baker's yeast, which is approximately 86% of LA production using YE. Using SCJ as substrate, the replacement of YE for baker's yeast decreased the LA production from 210.9 g/l to 178.2 g/l, around 16% lower.

Table 2-16 Lactic acid production using baker's yeast as nitrogen source

Condition	Lactic acid (g/l)
150 g/l of total sugar (HPPW) e 10 g/l of YE	134.1 ± 0.5
150 g/l of total sugar (HPPW) e 10 g/l of baker's yeast	113.0 ± 0.2
220 g/l of total sugar (SCJ) e 15 g/ l of YE	210.9 ± 0.4
220 g/l of total sugar (SCJ) e 15 g/ l of baker's yeast	178.2 ± 0.5

Altaf et al. (2007) studied the influence of the replacement of peptone and yeast extract for inexpensive nitrogen sources (red lentil-RL, and Baker's yeast cells-YC) and starch on LA production by *Lactobacillus amylophilus* GV6. Maximum LA production of 13.5 g was obtained with RL 0.8%, YC 1% and for 48 h, with 92% yield.

Despite the decrease in LA production of 14% and 16%, replacing the nitrogen source can be justified by the cost of each source, while yeast extract costs R$ 22.00/kg, the inactivated yeast costs about R$ 1.00/kg, which means a reduction of 95% of the cost of nitrogen source. This fact must be analyzed very carefully by the industry.

2.3.4 Lactic acid production in non-sterile conditions

Studies in non-sterile condition of fermentation have been development in order to decrease LA production costs of sterilization process. In this study, *L. pentosus* was cultivated in HPPW at 30ºC and under non-sterilized conditions producing 114 g/l of LA, which represents 76% of yield.

Studies for LA production by non-sterilized fermentation process have been developed using thermo-tolerant *Bacillus* due to its capacity at higher fermentation temperature. Ouyang et al. (2013) using lignocellulosic hydrolyzates obtained yield of 74.5% and LA concentration of 56.37 g/l (batch fermentation) and 75.03 g/l (fed-batch fermentation). In another study, *Bacillus coagulans* produced high amount of lactic acid (98.1 g/l and yield of 98%) from excess sludge hydrolysate to substitute yeast extract (Ma *et al.*, 2014). The result of LA production obtained in this study was higher than LA production results reported in the literature and in addition, it has an advantage of using lactic acid bacteria instead of *Bacillus* bacteria, because of the lower fermentation temperature used.

2.3.5 Lactic acid production during the optimization process in hydrolyzed potato processing waste and sugar cane juice

Optimization steps of production of LA in PHP and SCJ involved several steps. The optimization steps with their earnings in the process are briefly presented in Table 2-17 and Table 2-18. It can be observed a significant enhancement in LA production in each optimization step towards from the initial conditions of fermentation. In the end of optimization process, it was obtained 150 g/l of LA using HPPW and 225 g/l of LA using SCJ. The initial sugar concentration was higher in SCJ, because of this the concentration of LA in this substrate was higher than HPPW.

Table 2-17 Summary of the optimization steps of lactic acid production using HPPW as substrate

Optimization Step	LA concentration	LA productivity
Screening of nutrients	44 g/l	0.6 g/l.h
Optimization	135 g/l	1.9 g/l.h
Kinetics in Erlenmeyer flasks	150 g/l	1.6 g/l.h
Kinetics in STR	110 g/l	2.4 g/l.h

Table 2-18 Summary of the optimization steps of lactic acid production using SCJ as substrate

Optimization Step	LA concentration	LA productivity
Screening of Nitrogen Source	90 g/l	1.3 g/l.h
Optimization	199 g/l	2.8 g/l.h
Kinetics in Erlenmeyer flasks	225 g/l	2.3 g/l.h
Kinetics in STR	176 g/l	3.7 g/l.h

Table 2-19 present recent studies on LA fermentation and the type of optical isomer obtained according to the used microorganism. The results obtained on this fermentation process are consistent with the results described in the literature (150 g/l and 225 g/l). LA Productivity obtained using SCJ was 3.7 g/l.h, it is similar productivities obtained by Moon et al. (2012) and Wang et al. (2011).

The analysis of the optical isomer made by D-lactate kit showed that the LA produced by the *L. pentosus* B-227 in optimized conditions with both substrates is composed by L-lactic (95%) acid and D-lactic acid (5%).

Table 2-19 Studies on lactic acid production by fermentation

Microorganism	Substrate	La Production (g/l)	Yield	Productivity (g/l.h)	Optical Isomer	Reference
Enterococcus mundtii QU 25	Glucose/xylose	129	0.785	0.768	L (-)	(Abdel-Rahman et al., 2015)
Bacillus sp. WL-S20	Peanut meal and glucose	225	0.993	1.04	L (100%)	(Meng et al., 2012)
Lactobacillus paracasei	Glucose	192	0.96	3.99	L (96.6%)	(Moon et al., 2012)
Escherichia coli strain CICIM B0013-070	Glicerol	111.5	0.64	3.45	D (99.9%)	(Tian et al., 2012)
Lactobacillus agilis	Soybean vinasse	138	0.849	0.863	L (90-93%)	(Karp et al., 2011)
Sporolactobacillus sp. strain CASD	Peanut meal and glucose	207	0.93	3.8	D (99.3%)	(Wang et al., 2011)

2.4 CONCLUSIONS

The study demonstrated that alternatives proposed for LA production have potential for an industrial production process. The yield of LA production was higher using potato processing waste and sugar cane juice as substrate. The HPPW requires an acid pre-treatment under high temperature to hydrolyze the starch to glucose and maltose. Contrarily, sugarcane juice does not require pre-treatment.

The replacement of nitrogen source (yeast extract for baker's yeast) provided high LA concentration and high yield. The use of inactivated yeast demonstrated good alternative to reduction of LA production cost, because the inactivated yeast can be obtained from alcoholic fermentation, brewing fermentation as well as baker's yeast.

The fermentation in nonsterile conditions eliminates the step of sterilizing the culture medium thereby reducing the cost of LA production process.

CHAPTER 3 – SEPARATION AND RECOVERY PROCESS OF LACTIC ACID PRODUCED BY FERMENTATION

ABSTRACT

Lactic acid (LA), $C_3H_6O_3$, a naturally occurring organic acid, presents diverse applications mostly in food industry, as well as in pharmaceutical, chemical industries and for the production of poly(lactic acid) (PLA). The production of LA by fermentation offers the advantage of producing optically high pure LA. However, the disadvantage is the impurities such as residual sugar compounds, color and other organic acids that affect downstream processes increasing the costs of purification steps. The separation and recovery process of LA from fermented broth was developed to obtain a purified LA for the further studies of polymerization. The developed process consisted in heating the fermented broth, Then a centrifugation step was conducted for removal of the cells and suspended solids. A clarification step was included with powered activated carbon with further precipitation at low temperature and acidification of calcium lactate to convert to LA. The process was effective for removal of contaminants that were present in the fermentation medium. Final concentration of LA in aqueous solution was 416 g/l and a yield of 51%. Some alternatives were proposed to increase the yield.

KEYWORDS: Lactic acid, Calcium lactate, Powered activated carbon.

3.1 INTRODUCTION

The production of LA by fermentation offers the advantage of producing optically high pure LA after selecting an appropriate strain. Presently, almost all LA produced globally is manufactured by fermentation routes (Abdel-Rahman *et al.*, 2011). The production of LA by microorganisms is made by lactic acid bacteria and some filamentous fungus (Madhavan Nampoothiri *et al.*, 2010). Pure isomers are more valuable for specific industrial application than the racemic form (Abdel-Rahman *et al.*, 2011).

The disadvantage of fermentation is that the fermentation broth contains a large number of impurities such as residual sugar compounds, colour and other organic acids. Fermentation conditions also determine the quality of broth and suitability of downstream processing options (Joglekar *et al.*, 2006; Ghaffar *et al.*, 2014). Cheap wastes contain large number of impurities in larger quantities and the addition to the medium affect downstream processes because

removal of impurities can significantly increase the costs of purification steps (Castillo Martinez *et al.*, 2013). Techniques have been reported for impurities removal from the fermented broth by extraction, adsorption, electrodialysis, esterification, distillation, ultrafiltration and chromatographic methods (Joglekar *et al.*, 2006).

Downstream process for recovery and purification of LA consists in filtering the broth containing calcium lactate to remove cells. After that steps of, active carbon adsorption, evaporation and acidification with sulphuric acid to get lactic acid and calcium sulphate. The insoluble calcium sulphate is then removed by filtration. Then LA is obtained by hydrolysis, esterification and distillation (Wasewar, 2005). Activated vegetable carbon is used to bleach the calcium lactate and lactic acid obtained after acidification. Furthermore, studies used activated carbon in order to adsorb LA. (Bayazit *et al.*, 2011) compared the adsorption efficiency of LA onto activated carbon and Amberlite IRA-67. For adsoption of LA, activated carbon was less efficient than Amberlite IRA-67.

The aim of this study was to develop the separation and recovery process of LA from fermented broth and use it in further studies for polymerization reaction.

3.2 MATERIAL AND METHODS

3.2.1 Material

Lactobacillus pentosus NRRL B-227 was cultivated in MRS (Man–Rogosa–Sharpe) broth at 35ºC during 24 hours. After incubation, glycerol was added to the culture and the broth was stored at -20 ºC and periodically renovated.

In clarification study, powdered activated carbon was utilized (C118-CB) that was obtained from Carbomafra S/A (Brazil).

3.2.2 Lactic acid production by fermentation

The fermentation for lactic acid production was carried out in a 5 l stirred tank reactor containing 160 g/l of total sugar from hydrolyzed potato starch, 10 g/l of yeast extract and 80 g/l of CaCO3, inoculum rate of 10%.

3.2.3 Recovery and purification of lactic acid

Recovery and purification of LA from fermented broth was developed according to steps demonstrated in Figure 3-1.

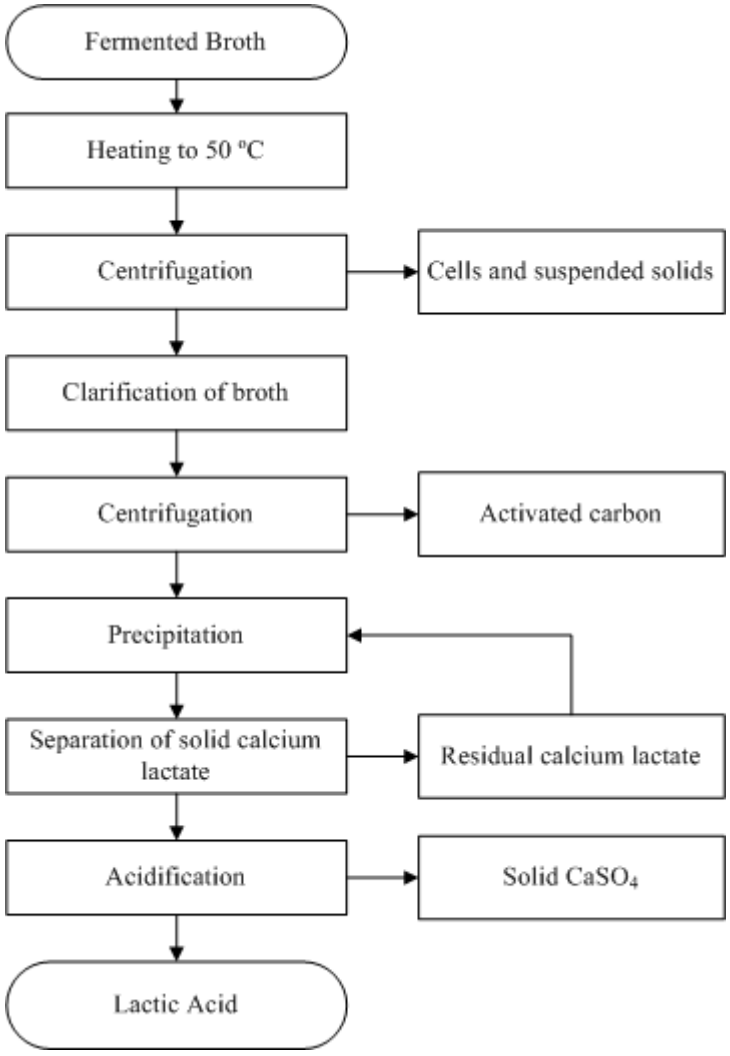

Figure 3-1 Flow sheet of developed steps of LA separation and recuperation processes

At the end of fermentation in STR, the calcium lactate presented in fermented broth precipitated. In order to dissolve the calcium lactate, the fermentation broth was heated to 50°C. Then the broth was centrifuged at 1800 x g for 20 minutes (Centribio, model 80-2B) to remove suspended solids and bacterial cells. The LA concentration and yield were determined.

The supernatant of centrifugation process was submitted to a clarification that was conducted with powdered activated carbon under different conditions with the support of a full factorial experimental design $2^{(4-0)}$. The studied variables were temperature, agitation, time and concentration of activated carbon (Table 3-1). The experiment was conducted in 125 ml Erlenmeyer flasks with a volume of 50 ml. Incubation was made in "water bath" with agitation (Ethiktechnology, model 501-D). The activated carbon was removed from the broth at temperatures between 30 and 50°C by centrifugation at 1800 x g for 20 minutes.

Table 3-1 Complete factorial experimental design for fermented broth clarification study

Independent variable	Levels		
	-1	0	+1
Temperature (ºC)	30	40	50
Agitation (rpm)	0	50	100
Time (minutes)	5	15	25
Concentration of activated carbon (%) (w/v)	5	10	15

After removing the activated carbon, the clarified broth was subjected to precipitation, thus obtaining the solid calcium lactate. The precipitation was carried out in a Beaker glassware at low temperature (around 4ºC) at static conditions during 24 hours. The solid salt was recovered by filtration and the filtered broth containing part of the calcium lactate, so as it could be returned to the recovery process.

The solid calcium lactate was converted to LA using sulfuric acid at different concentrations (1, 2, 3, 4 and 5 mol/l). The acidification was performed in a Beaker glassware under magnetic agitation. The yield of conversion was determined.

3.2.4 Analytical methods

Fermented broth was acidified with sulphuric acid to convert calcium lactate to lactic acid. After, the broth was centrifuged at 1800 x g for 20 minutes. The supernatant was analyzed in high performance liquid cromatograph (HPLC).

Lactic acid and reducing sugars were analyzed by high performance liquid chromatography (HPLC) (Shimadzu LC 10AD, detector RID 10A), using the Aminex HPX-87 H column, 5mM H_2SO_4 mobile phase , 0.6 mL/min, at 60ºC. Standards for chromatography analyses were L-lactic acid 99% (Sigma Aldrich), D-glucose and D-maltose.

3.3 RESULTS AND DISCUSSION

The centrifugation of fermented broth removed bacterial cells and suspended solids including $CaCO_3$ excess. Before centrifugation, the concentration of LA was 100 g/l and after centrifugation was 94.5 g/l. The yield of this step was 94.5%.

The clarification step using powdered activated carbon (PAC) consisted of removing juice color, organic substances, because the PAC has a large contact surface that promotes adsorption components. The first experiment was conducted at room temperature (around 20 ºC) and it was observed that calcium lactate precipitates in contact with the PAC. Therefore,

the experiments were conducted with controlled temperature above 30°C according to a factorial experimental design, which results are presented in Table 3-2. It can be observe that there was no significant difference on LA concentration in all studied conditions. However, there was significant difference in yield. The yield of clarification was calculated considering the initial volume and final volume of the broth. Lower yields, around 60%, were obtained when using higher amounts of PAC.

Table 3-2 Factorial experimental design for clarification of fermented broth

Run	Temperature (ºC)	Agitation (Rpm)	Time (Minutes)	Concentration of Active Carbon (%)	Lactic Acid (g/l)	Yield
Control	-	-	-	-	94.5	100%
1	50	100	25	15	120.4	64%
2	50	100	25	5	99.1	77%
3	50	100	5	15	93.5	60%
4	50	100	5	5	95.6	77%
5	50	0	25	15	95.5	60%
6	50	0	25	5	94.5	73%
7	50	0	5	15	96.1	60%
8	50	0	5	5	95.1	80%
9	30	100	25	15	93.0	60%
10	30	100	25	5	94.9	67%
11	30	100	5	15	97.9	64%
12	30	100	5	5	91.9	83%
13	30	0	25	15	105.2	60%
14	30	0	25	5	97.6	73%
15	30	0	5	15	98.0	64%
16	30	0	5	5	91.2	77%
17 (C)	40	50	15	10	98.4	75%
18 (C)	40	50	15	10	99.6	70%
19 (C)	40	50	15	10	101.8	72%

The Pareto chart (Figure 3-2) shows that among the studied independent variables in this experiment only the variable concentration of PAC presented significant influence on LA adsorption process yield ($p<0.05$) with a negative effect The variable time did not influence significantly on process yield at $p<0.05$; however, there was significant difference at $p<0.1$. The other idenpendent variables temperature and agitation did not influence on process yield in the studied range.

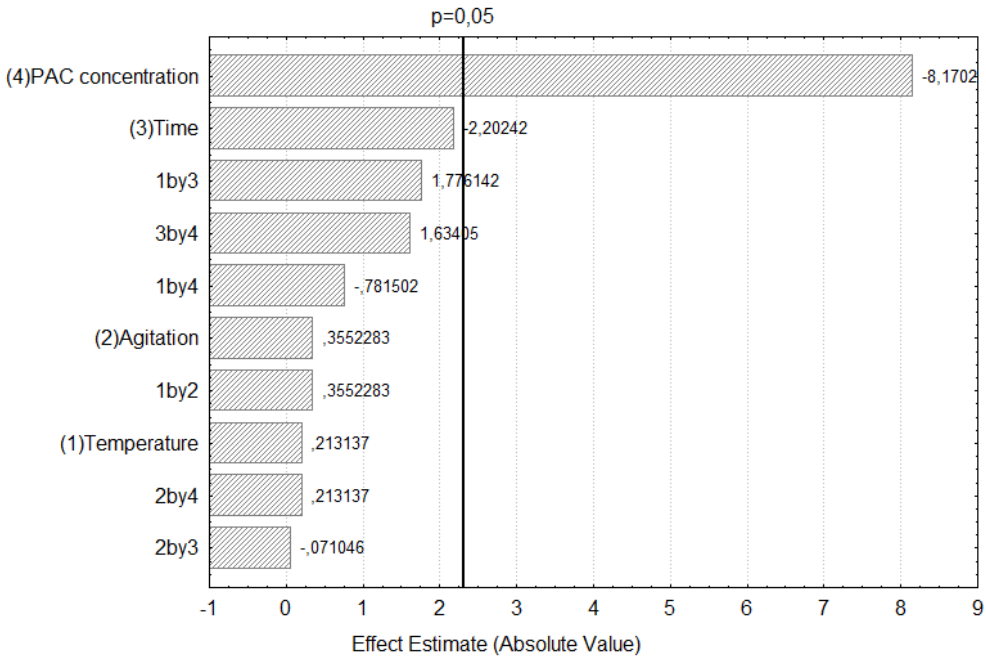

Figure 3-2 Pareto chart of the clarification study of LA fermented broth. R^2=0.907

Clear differences could be observed related to the color of the broth (Figure 3-3, Figure 3-4 and Figure 3-5). The clarified samples 1 and 9, showed lighter colors. The conditions for both runs were 100 rpm, 25 minutes and 15% of PAC, the difference between these runs was the incubation temperature, 50°C for run 1 and 30°C for run 9. Therefore, the condition of essay 9 was chosen for the clarification stage 30° C, 100 rpm, 25 minutes and 15% of PAC.

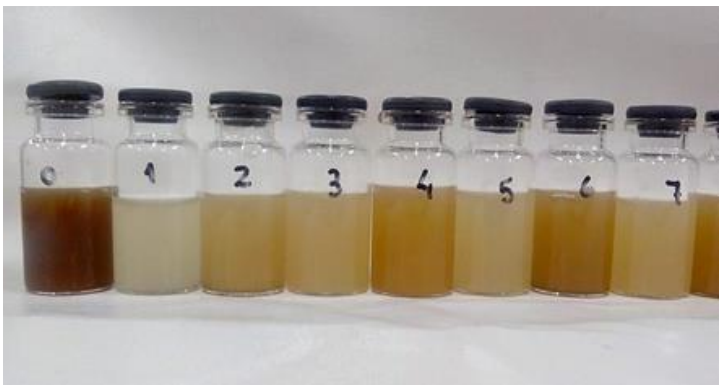

Figure 3-3 Samples after clarification of LA fermentation medium. From essay control (0) to essay 7.

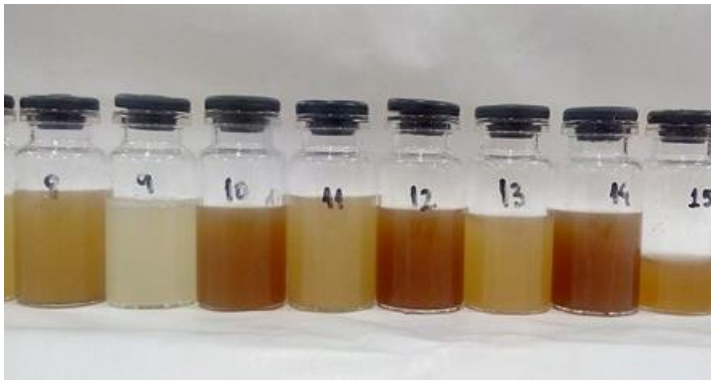

Figure 3-4 Samples after clarification of LA fermentation medium. From essay 8 to essay 15.

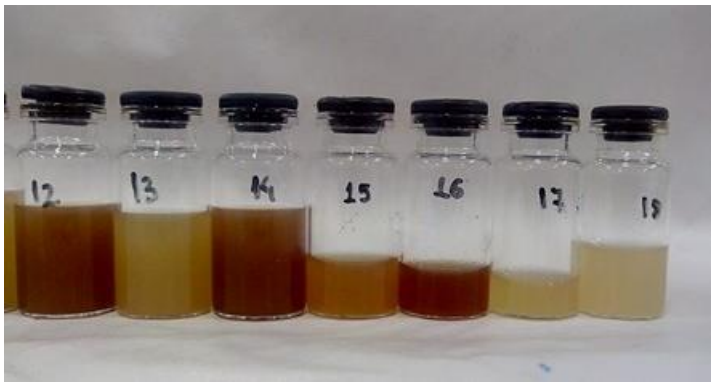

Figure 3-5 Samples after clarification of LA fermentation medium. From essay 12 to essay 18.

The process of precipitation at low temperature under static conditions, provided the precipitation of 75% of calcium lactate present in the clarified broth (Figure 3-6).

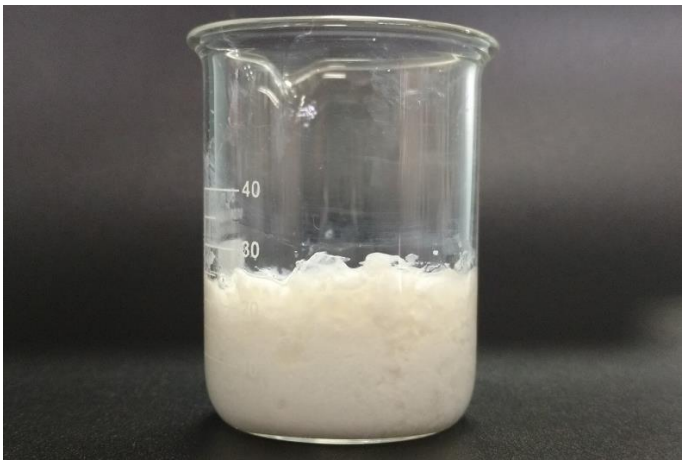

Figure 3-6 Solid calcium lactate obtained after precipitation process

The conversion of calcium lactate to LA was performed using H_2SO_4 at different concentrations (1, 2, 3, 4 and 5 mol/l) (Equation 3-1) and the yield of conversion was analyzed

(Table 3-3). When the concentration of H_2SO_4 was increased, the yield of acidification decreased because of the amount of water present in reaction decreased. This reaction must be in solution to occur. The main objective was to obtain LA containing a small amount of water because water influences the LA polymerization process.

$$Ca(C_3H_4O_2)_2 + H_2SO_4 \rightarrow 2\ C_3H_6O_3 + CaSO_4 \quad (3\text{-}1)$$

According to Table 3-3, the highest concentration of LA (495 g/l) was obtained with a yield of 84%, with 5 mol/l H_2SO_4.. A yield of 100% was obtained using 2 mol/l H_2SO_4, but the concentration of LA was 238.9 g/l. because of amount of water present in solution of H_2SO_4. However, for use in polymerization, the concentration of LA should be at least 2 or 3 times.

By using 4 mol/l H_2SO_4 the achieved yield was 88% and this yield was close to that obtained by Min et al. (2011), who achieved 92% of yield using the $Ca(LA)_2/H_2SO_4$ at a molar ratio of 1:1 and the pH higher than the pKa value.

Table 3-3 Acidification of calcium lactate using different concentration of H_2SO_4

H_2SO_4 (mol/L)	Experimental LA (g/l)	Theoretical LA (g/l)	Yield (%)
1	123.6	117.6	105%
2	238.9	235.3	102%
3	333.6	353.1	94%
4	416.1	470.6	88%
5	495.0	588.2	84%

The quality of LA and the yield of recuperation process is usually very important. The separation and recovery process is one of the bottlenecks to turn the production of LA economically viable (Wasewar, 2005). These steps can represent up to 50% of total costs of LA production (Chaudhuri and Phyle, 1992; Eyal and Bressler, 1993).

According to Table 3-4, it is possible to observe the partial yields of each step of the LA separation and recovery process. The final yield of the process is 51%, which is considered a low yield, and consequently, the production cost of LA increases. In this process, clarification and precipitation of calcium lactate contributed to the low yield. Alternatives can be proposed to increase the yield such as the recirculation of the supernatant after the precipitation process due to presence of 25% of calcium lactate and the recovery of calcium lactate adsorbed in the powered activated carbon.

Table 3-4 Yield of separation and recuperation process of lactic acid

Process	Condition	Yield
Centrifugation	1800 x g for 20 minutes	95%
Clarification	50 ºC, 100 rpm, 25 minutes and 15% of PAC (w/v)	64%
Precipitation	Low temperature and static mode	75%
Acidification	H_2SO_4 4 mol/l	88%
Total Yield of process		**51%**

3.4 CONCLUSIONS

The process of LA separation and recovery of the fermentation broth was effective for removing contaminants and, at the end of the process, lactic acid was achieved n aqueous solution, around 416 g/l. After all steps of recovery and purification processes the yield was 51%, which represents a low yield, which means that almost 50% of lactic acid produced by fermentation was lost. The LA recovery in this process can be applied on polymerization process of poly(lactic acid).

CHAPTER 4 – LACTIC ACID POLYCONDENSATION BY CONVENTIONAL AND MICROWAVE HEATING PROCESS

ABSTRACT

Poly (lactic acid) (PLA) is a polyester, which has a predominant role as biodegradable plastic. It is mainly applied in packaging, textile, medical and pharmaceutical products. It can be obtained from direct polycondensation of lactic acid and by ring-opening polymerization (ROP) of lactide. In this chapter, the polymerization of lactic acid was developed by polycondensation in two steps, azeotropic dehydration and direct polycondensation. The study of polymerization was carried out using as momoner commercial lactic acid and fermented lactic acid, moreover two different heating systems were studied the conventional and the microwave heating. The conventional process was conducted in a heating system that was connected to a distillation unity under reduced pressure. The first step consisted of the study of different catalysts (Tin 2-ethylhexanoate and zinc lactate) and solvents (toluene and xylene). In a second step, the zinc lactate and toluene previously catalyst and solvent, respectively selected, were studied in a factorial experiment design to evaluate the time of dehydration, toluene/LA ratio, time of polycondensation and catalyst/LA ratio. A polymer with 6330 g/mol of molecular weight and 61% of yield was obtained from commercial lactic acid after 2 hours of azeotropic dehydration, 1.5 toluene/LA ratio, 10 hours of polycondensation and 0.0006 catalyst/LA ratio. The polymer obtained from fermented lactic acid presented a molecular weight of 2360 g/mol after 8 hours. Microwave heating process provided a higher yield, 79% and 76% with commercial and fermented lactic acid respectively. Nevertheless, the polymer's molecular weight was lower than that obtained from conventional process, 2071 g/mol for commercial lactic acid and 1450 g/mol for fermented lactic acid.

KEYWORDS

Poly(lactic acid); azeotropic polymerization; microwave heating system

4.1 INTRODUCTION

Poly(lactic acid) (PLA), which is a biodegradable polymer obtained from lactic acid polymerization , has several applications in packaging, textile., Furthermore, PLA has been utilized as medical material and drug delivery system (Gupta and Kumar, 2007; Madhavan Nampoothiri *et al.*, 2010; Lasprilla *et al.*, 2012).

PLA can be obtained by direct polycondensation of lactic acid and by ring-opening polymerization (ROP) of the lactide. Commercially, PLA has been obtained by ROP, which leads to high polymer's molecular weights, however this process involves high costs due to the

purification process of the lactide (Leja and Lewandowicz, 2010). The direct polycondensation of lactic acid results in a low molecular weight PLA, therefore, studies using azeotropic condensation and solid-state polimerization methods have been developed in order to increase the molecular weight of the polymer.

The direct polycondensation method has a critical point, which is the removal of water that is formed during the reaction due to the high viscous reaction mixture. It is essential to ensure the water withdrawal to obtain high molecular weight polymers because this water can enhance the hydrolysis of formed polymer (Gupta and Kumar, 2007; Marques *et al.*, 2010; Södergärd and Stolt, 2010). The improvement of water removal can be obtained with, an azeotropic polymerization that is conducted using an organic solvent, which removes the water azeotropically. In this case, solvent is treated with a drying agent and then recycled back in the reaction. The used reaction temperature is below the melting point of the polymer, which prevents depolymerisation and racemisation reactions during polymerization (Madhavan Nampoothiri *et al.*, 2010; Gupta and Kumar, 2007).

Conventional heating is carried out by conduction and the heating system consists of an external heat source, such as an oil bath. The heat transfer is low, which can result in an inefficient control of the temperature of the reaction mixture. Microwave (MW) heating process has been attracted attention in recent years due to its potential to accelerate the chemical reaction and drastically reduce the production energy, when compared with the conventional conductive heating processes. Furthermore, it reduces the number of side reactions, increases yields and improves reproducibility (Kappe, 2004).

MW can directly heat materials via a dielectric interaction between them (Nakamura *et al.*, 2010). Molecules exhibiting a permanent dipole moment will be aligned to the applied electromagnetic field generating heat because of friction and collision of molecules (Hoogenboom and Schubert, 2007). The high temperatures attained and the ability to work under high pressure conditions for relatively short times make reactions faster than under conventional thermal conditions and greater yields are usually obtained (Kappe, 2004; Sosnik *et al.*, 2011).

This chapter presents the study of polymerization of commercial and fermented lactic acid, including the analysis of influence of different heating systems, conductive heating and microwave heating system.

4.2 MATERIAL AND METHODS

4.2.1 Material

Commercial lactic acid 85% (Vetec, Brazil), lactic acid obtained by fermentation, zinc lactate (Synth, Brazil), toluene (Neon, Brazil), acetone (Neon, Brazil), Tin(II) 2-ethylhexanoate ($Sn(Oct)_2$) (Sigma Aldrich, USA), butanediol (Vetec, Brazil) and xylene (Vetec, Brazil).

The monomer lactic acid 85-90% (Alfa Aesar, Belgium) and lactic acid obtained by fermentation were used in polymerization process by microwave, as well as zinc lactate (Synth, Brazil), toluene (Anachemia, Canada) and acetone (Fisher Scientific, United States of America).

4.2.2 Lactic acid production by fermentation

Lactic acid production is carried out by fermentation in a 5 l stirred tank reactor containing 160 g/l of total sugars from hydrolyzed potato processing waste, 10 g/l of yeast extract, 80 g/l of $CaCO_3$ and inoculum rate of 10% (v/v).

The fermented broth is centrifuged to remove cells and calcium carbonate. After that, adsorption using active carbon is carried out. The calcium lactate, which is in the clarified broth, is precipitated at 4ºC and then acidified with H_2SO_4 in order to convert calcium lactate into lactic acid. The amount of each isomer (D or L) was determined by D-Lactate Colorimetric Assay Kit (Sigma Aldrich, USA).

4.2.3 Lactic acid polymerization by conventional heating method

4.2.3.1 Screening of catalysts and solvents for polymerization process

Polymerization process was performed in two different steps: water removal from LA by azeotropic dehydration process and polycondensation process under reduced pressure. The first step was based on an adaptation of the azeotropic polymerization system, which consists on the distillation of lactic acid for 2−3 h at 130°C with organic solvent and catalyst at reduced pressure to remove most of the condensed water in a Dean Stark trap. After, the solvent returns to the vessel by molecular sieves during 30−40 h at 130°C (Averous, 2008). The polymerization method developed in this work is different from the azeotropic polymerization described in the literature because there is no recirculation of the solvent after the distillation of lactic acid.

Different catalysts (Tin 2-ethylhexanoate or zinc lactate) and solvents (toluene or xylene) were tested, as well as the polymerization that was also carried out without the use of catalyst and solvent.

The first step was carried out in a 125 mL flask containing 25 mL of 85% LA that was connected to a reflux system in the presence of the catalyst at the defined ratio 1:1000 (w/w, catalyst/monomer) and solvent 1:1 (v/v). The process occurred under magnetic agitation at 110-120ºC for 2 hours. Afterwards, the water was removed azeotropically using a Dean-Stark apparatus under reduced pressure at solvent boiling point.

The second step was the polycondensation reaction that was conducted in a 125 mL flask adapted to the distillation system. The flask was heated to 180ºC for 4 hours using an oil bath and magnetic stirring. During the reaction, the pressure was reduced using vacuum pump (Figure 4-1).

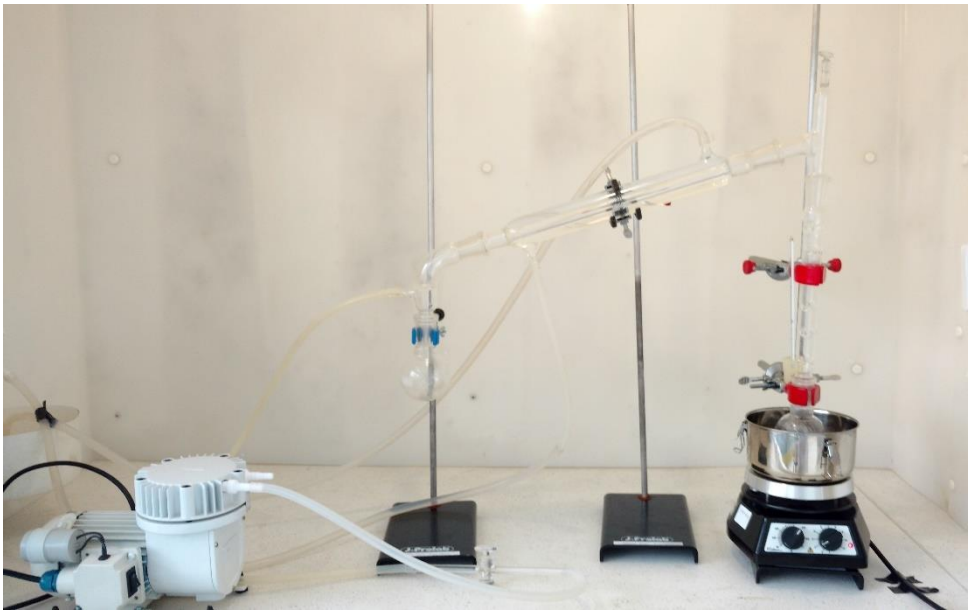

Figure 4-1 Polycondensation process under reduced pressure

4.2.3.2 Development of the polymerization process with a $2^{(k-1)}$ factorial experimental design

A study was carried out to evaluate the influence of some polymerization conditions, using a $2^{(k-1)}$ factorial experimental design with toluene as solvent and zinc lactate as catalyst. The independent variables toluene/LA ratio, time of dehydration, catalyst concentration and time of polymerization were studied at different levels resulting in 8 runs and 3 central points (Table 4-1).

For each experiment, 25 g of LA were added to a 125 mL flask that was connected to a reflux system in the presence of the defined catalyst and solvent, under magnetic agitation at 110-120 ºC. The water was removed azeotropically using a Dean-Stark apparatus under reduced pressure at 110-120ºC. The second step, the polymerization reaction, was conducted in a 125 mL flask that was adapted to distillation system. The flask was heated to 180ºC for 4 hours using an oil bath and magnetic stirring. During the reaction, the pressure was reduced using vacuum pump.

The dependent variables were: the yield (%) and molecular weight. The molecular weight was determined by the Viscosimetric Molecular Weight Method (Mv).

Table 4-1 Polymerization conditions of LA by conventional heating method - $2^{(k-1)}$ Factorial experimental design

	LEVELS		
	-1	0	+1
Toluene / LA ratio (v/v)	0,5/1	1/1	1,5/1
Catalyst/LA ratio (m/m)	0,0004/1	0,001/1	0,0016/1
Time of dehydration (hours)	2	4	6
Time of polymerization (hours)	4	7	10

4.2.3.3 Polymerization of lactic acid obtained from fermentation

The lactic acid obtained from fermentation was polymerized in a 125 mL flask connected to a reflux system using zinc lactate (zinc lactate/monomer - 0,0004/1 – w/w) and toluene (toluene/monomer - 1,5/1 – v/v) under magnetic agitation at 110-120ºC for 2 hours. The water was first removed azeotropically using a Dean-Stark apparatus under reduced pressure at 110-120 ºC. After that, the polymerization reaction was conducted in a 125 mL flask adapted to a distillation system. The flask was heated to 180ºC for 8 hours using an oil bath and magnetic stirring. During the reaction, the pressure was reduced using a vacuum pump.

4.2.3.4 Azeotropic dehydration of lactic acid - three-level full factorial design

The process was carried out with the support of a 3^2 full factorial experimental design using three levels and two independent variables (toluene/LA ratio and time) (Table 4-2). 20 g of LA and toluene were added to a 125 mL flask connected to a reflux system under stirring at 110-120ºC. Then, toluene was distillated using a Dean-Stark apparatus under reduced pressure.

Table 4-2 Azeotropic dehydration of lactic acid - 3^2 full factorial experimental design

	LEVELS		
	-1	0	+1
Toluene/ LA ratio (v/v)	1:1	2:1	3:1
Time (hours)	2	4	6

4.2.4 PLA Synthesis in Microwave Oven

Microwave heating polymerization of commercial and produced lactic acid was developed at *Institute National de la Recherche Scientifique*, Quebec city, Canada, under the supervision of Dr. Satinder Kaur Brar.

Microwave heating was performed in a microwave MARS 5 (CEM Corporation, North Carolina, United States of America) with 1600 W maximum power output. The polymerization reaction was carried out in a sealed 100 ml Teflon® reactor vessel (HP-500, 500 psig material design pressure and 260 °C). (Figure 4-2).

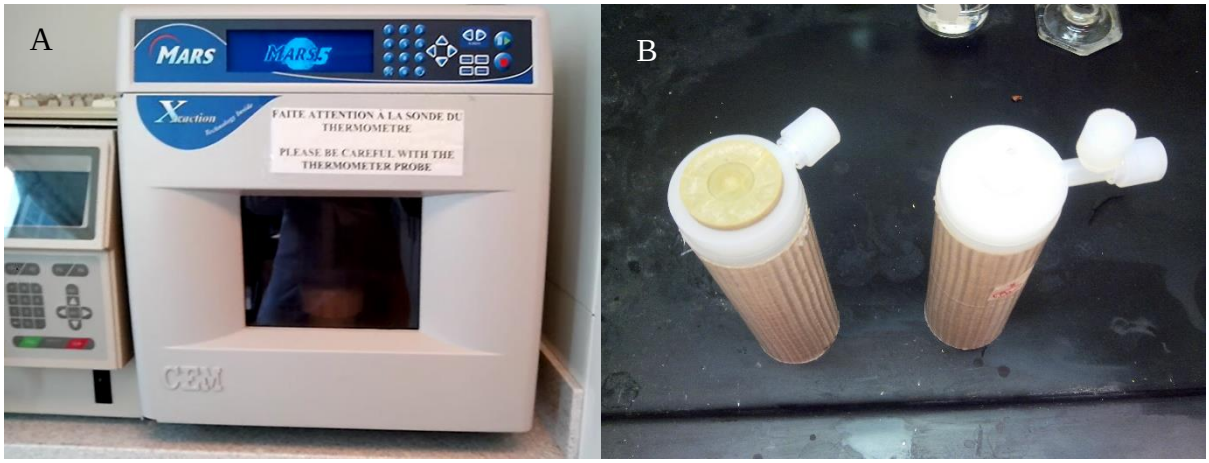

Figure 4-2 Microwave used at polymerization process. A) MARS 5 (CEM Corporation). B) Teflon® reactor vessel (HP-500).

4.2.4.1 Water removal by azeotropic distillation

The water present on lactic acid (commercial and produced by fermentation) was removed before the polymerization reaction in microwave. Azeotropic distillation consisted of a heating step of the lactic acid, in the presence of toluene (1,5:1 v/v), in a Buchi® rotary evaporator (model R-215) at 90ºC and 15 inHg (0.5 atm) of pressure during 2 hours.

4.2.4.2 Microwave polymerization optimization

Polymerization optimization essays in microwave were carried out according to a Box-Behnken experimental design with the analysis of different levels of power, reaction temperature and time (Table 4-3). Essays were conducted at atmospheric pressure. For each experiment, 15 g of lactic acid and 0.015 g of zinc lactate were employed. The dependent variables were the yield (%) and molecular weight determined by the Viscosimetric Molecular Weight Method (Mv).

Table 4-3 Optimization of lactic acid polymerization by microwave heating process - Box-Behnken experimental design

	LEVELS		
	-1	0	+1
Power (W)	100	200	300
Temperature (°C)	140	160	180
Time (minutes)	60	90	120

4.2.4.3 Polymerization of lactic acid obtained from fermentation

The lactic acid obtained from fermentation was submitted to the same conditions of polymerization as described for the polymerization of commercial lactic acid (Section 4.2.4.2). Both techniques were then compared based on the characteristics of the obtained polymers, yield and molecular weight.

4.2.5 Characterization Techniques

After polymerization, the polymer was cooled to room temperature and dissolved in acetone. The precipitation of the polymer was carried out in water and the solid was dried. The yield of polymerization was determined by the weight ratio between the precipitated polymer and the monomer (Equation 4-1).

$$Yield(\%) = \frac{Polymer\ (g)}{Monomer\ (g)} \times 100 \tag{4-1}$$

The molecular weight determination was carried out by Gel Permeation Chromatography (GPC) or Viscosimetric Molecular Weight (Mv). Chromatographic analysis

of gel permeation was conducted in a Waters 1515 system, equipped with a refractive index detector Model 2487. Two columns TSK-Gel HXL in series, G2000 and G1000, were employed whose exclusion limits correspond to 4000 and 1500, respectively. The analyzes were performed at 45°C using tetrahydrofuran (THF), previously deaerated, as mobile phase at a flow rate of 0.8 ml/min at 40°C. The injection procedure was performed using an autosampler 2707, whose injection volume was 10 μL. The apparent molecular weight was calculated from the standard monodisperse polystyrene retention volume calibration curve in which the molar weight range from 50000 to 436.

The viscosimetric molecular weight (Mv) was determined by viscosimetry using a Cannon–Fenske N50 capillary viscometer placed in a water bath at 25°C. The polymers were dissolved in chloroform at concentrations ranging from 1 to 4 g/dL. The intrinsic viscosity [η] data were converted into molecular weights with the Mark-Houwink equation (Equation 4-2), where for chloroform at 25 ºC, values of k and a are 5.45×10^{-4} and 0.73, respectively (Perego *et al.*, 1996).

$$[\eta] = K \times M_v^a \tag{4-2}$$

In the study of dilute solutions of polymers, the relative viscosity (η_r) was determined by the solution viscosity compared to pure solvent viscosity. To determine the value of this viscosity, the flow time of solvent (t_0) and solution (t) were measured on the same viscometer. Whereas the density of the diluted solutions was practically equal to that of the pure solvent, the relative viscosity could be defined by the Equation 4-3 (Lucas *et al.*, 2001).

$$\eta_r = \frac{t}{t_0} \tag{4-3}$$

The specific viscosity is defined as:

$$\eta_{sp} = \eta_r - 1 = \frac{t - t_0}{t_0} \tag{4-4}$$

The reduced specific viscosity (η_{redsp}) is defined by Equation 4-5, where c – polymer concentration (g/dl).

$$\eta_{redsp} = \frac{\eta_{sp}}{c} \tag{4-5}$$

Inherent viscosity (η_{inh}) is defined by Equation 4-6.

$$\eta_{inh} = \frac{\ln(\eta_r)}{c} \tag{4-6}$$

The intrinsic viscosity may be calculated plotting the inherent viscosity versus concentration and reduced specific viscosity versus concentration (Figure 4-3). To extrapolate the graph to zero concentration. The intrinsic viscosity (η) is the intercept of the line at zero concentration.

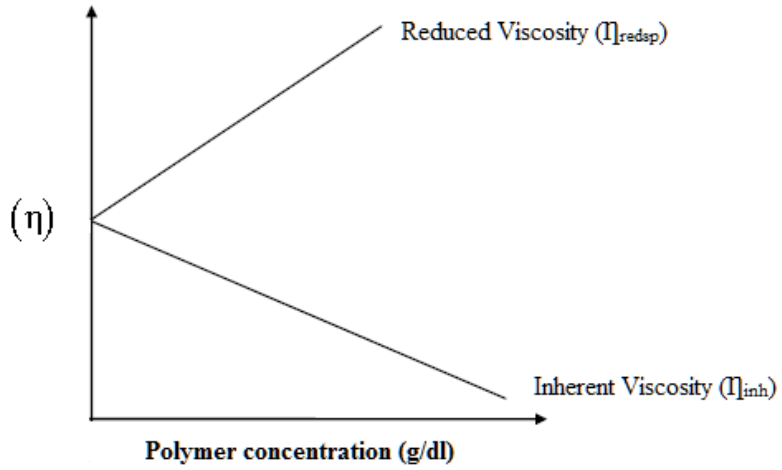

Figure 4-3 Graphic representation of intrinsic viscosity

Acid value was determined according to Jiménez-Bonilla et al. (2014). It was used 0.1 g of sample dissolved in acetone and titrated with 0.1 M KOH in isopropanol. As indicator was used phenolphthalein 1% in ethanol. Acid value is calculate by Equation 4-7, where v – volume of KOH solution (ml), M – concentration of KOH (mol/L) and m – mass of sample (g).

$$Acid\ value\ \left(\frac{gKOH}{g}\right) = \frac{v \times M \times 56.11}{m} \tag{4-7}$$

4.3 RESULTS AND DISCUSSION

4.3.1 Lactic acid polymerization by conventional heating method

4.3.1.1 Screening of catalysts and solvents for polymerization process

Molecular weight of polymers obtained using different catalysts and solvents are shown in Table 4-4. Polymerization reaction without the presence of catalyst and solvent (Run 1) resulted in a low molecular weight polymer, 930 g/mol. Achmad et al. (2009) obtained polymers with 90,000 g/mol using L-LA 60% under vacuum without catalyst and solvent. The time required was very long, 96 h, which represents 24 times higher than the time of polymerization in this study.

Table 4-4 Results of PLA polymerization using different catalyst and solvent

Run	Catalyst	Solvent	Mn
1	N	N	930±33
2	Sn(Oct)2	N	980±49
3	Zinc lactate	N	1830±146
4	N	Toluene	1000±2
5	Zinc lactate	Toluene	2530±134
6	Sn(Oct)2	Toluene	2110±44
8	N	Xylene	-

N – Not present.

The presence of catalyst and solvent during polymerization increased the molecular weight of polymer. The use of an organic solvent, which forms an azeotrope with water increases the molecular weight of the polymer because the water formed during the polycondensation synthesis is removed azeotropically (Madhavan Nampoothiri *et al.*, 2010; Gupta and Kumar, 2007; Enomoto *et al.*, 1994).

When zinc lactate and toluene were used as catalyst and solvent, respectively, for polymerization, a higher molecular weight was obtained, 2530 g/mol. A study developed by Schwach et al. (1998) demonstrated that zinc lactate is a good alternative to metal zinc and $Sn(Oct)_2$, because the attack of zinc metal by lactic acid generates zinc lactate, which act as the real catalyst during polymerization.

The polymer obtained using $Sn(Oct)_2$ and $Sn(Oct)_2$/toluene presented lower molecular weights, 980 g/mol and 2110 g/mol, respectively. High molecular weight PLA has been obtained using $Sn(Oct)_2$ as catalyst, however by ring opening polymerization (ROP) of lactide (Orozco *et al.*, 2014; Nanavati and Katiyar, 2010; Ganapathy *et al.*, 2007).

The use of xylene during polymerization resulted in a non-precipitation of polymer. Xylene has been used in polymerization of LA, Berger and Gregorova (2014) synthesized PLA by azeotropic condensation of L-LA in xylene and modified functional end groups of PLA by succinic anhydride and l-cysteine by the addition–elimination reaction. Yamada et al. (2014) investigated polymerization of LA in xylene catalyzed by scandium trifluoromethanesulfonate using a Dean–Stark apparatus under a conventional heating process and microwave heating process. Abiko et al. (2012) polymerized L-LA with carboxylic acid terminal groups using xylene and triphenylphosphonium trifluoromethanesulfonate under reflux during 20 hours.

Thus, for the next experiment it was selected zinc lactate as catalyst and toluene as solvent for polymerization process of lactic acid.

4.3.1.2 Development of the polymerization process with a $2^{(k-1)}$ factorial experimental design

The polymerization yield and polymer's Molecular Weight (Mv) was analyzed as a function of time of dehydration, toluene/LA ratio, time of polycondensation and catalyst/LA ratio (

Table 4-5). The highest yield of polymerization was obtained at run 3 and 5, representing 62% and 61%, respectively. These results are higher than those obtained by Orozco et al. (2007) who performed polycondensation of lactic acid using different kinds and amounts of tin based catalysts during 12 hours. The yields of reactions were from 32% to 54%.

Table 4-5 Results of polymerization according to $2^{(k-1)}$ factorial experimental design

Run	Time of Dehydration (Hours)	Toluene/La Ratio (V/V)	Time of Polymerization (Hours)	Catalyst/LA Ratio (W/W)	Yield (%)	Mv (g/mol)
1	6	1.5	10	0.0016	57	3100
2	6	1.5	4	0.0006	59	2910
3	6	0.5	10	0.0006	62	4070
4	6	0.5	4	0.0016	46	2330
5	2	1.5	10	0.0006	61	6330
6	2	1.5	4	0.0016	37	2270
7	2	0.5	10	0.0016	59	4550
8	2	0.5	4	0.0006	46	2020
9	4	1.0	7	0.001	54	2990
10	4	1.0	7	0.001	55	3060

11	4	1.0	7	0.001	52	2910

Statistical analysis of the factorial experimental design was carried out considering yield as dependent variable. The analysis of variance (Table 4-6) presented that only time of polymerization had significant positive effect on polymerization yield ($p<0.05$) and $R^2= 0.828$. During polycondensation process, due to side reactions, sub-products are produced such as lactide. These side reactions influence the polymerization yield, which means that high polycondensation yields result in low lactide production and low polymerization yields results in high lactide amounts (Garlotta, 2001; Orozco *et al.*, 2007).

Table 4-6 Analysis of variance (ANOVA) for polymerization yield. R^2=0.828

	SS	df	MS	F	p
(1) Time of dehydration	0.0055	1	0.0055	2.1812	0.2137
(2) Toluene/LA ratio	$1.25x10^{-05}$	1	$1.25x10^{-05}$	0.0049	0.9473
(3) Time of polymerization	0.0325	1	0.0325	12.8645	0.0231
(4) Catalyst/LA ratio	0.0107	1	0.0107	4.2241	0.1090
Error	0.0101	7	0.0025		
Total SS	0.0588	10			

In this experimental design, at levels studied, the highest molecular weight of polymer was 6330 g/mol, which corresponds to essay 5 (2 hours of dehydration, 1.5 of toluene/LA ratio, 10 hours of polymerization and 0.0006 catalyst/LA ratio). According to
Table 4-5, the increase of molecular weight is proportional to the increase of the time of polymerization reaction. The analysis of variance (Table 4-7) showed that only time of polymerization influenced significantly ($p<0.05$) the molecular weight of polymer. In this study, the time of polycondensation was considered short. Studies demonstrate that long time of polymerization are required to high molecular weight polymers, for example, 30 h of 2 steps direct polycondensation resulted in 33,300 g/mol (Pivsa-Art *et al.*, 2013). Even for ring opening polymerization of lactide, the time of polymerization required is long. Orozco et al. (2014) studied ROP for 24 h and Singla et al. (2012), from 34 h to 133 h.

Table 4-7 Analysis of variance (ANOVA) for viscosimetric molecular weight (Mv). R^2=0.759

	SS	df	MS	F	p
(1) Time of dehydration	956344.5	1	956344.5	1.0473	0.3639
(2) Toluene/LA ratio	334562	1	334562	0.3664	0.5776
(3) Time of polymerization	9069540.5	1	9069540.5	9.9318	0.0344
(4) Catalyst/LA ratio	1131400.434	1	1131400.434	1.2389	0.3280

Error	3652714.566	7	913178.6416
Total SS	15144562	10	

The amount of catalyst did not significantly influence, at the levels studied, on molecular weight and the reaction yield. In the literature, the amount of catalyst differs considerably. The amount of toluene and time of reflux did not influence the molecular weight and yield. However, Gupta and Kumar (2007) affirmed that the presence of solvent contributes for water removal from LA and water formation during the reaction of polycondensation, thus providing an increase on molecular weight.

The experiment conducted with LA produced by fermentation was carried out under the following conditions that promoted higher molecular weight and yield using commercial lactic acid as monomer: 2 hours of azeotropic dehydration, 1.5 of toluene/LA ratio and 0.006 catalyst/LA ratio), in different periods, 4 and 8 hours (Table 4-8). The increase of reaction time did not influence the yield, 55% e 56%, but caused the increase of molecular weight, from 1870 g/mol to 2370 g/mol. The molecular weight from fermented LA was lower than that of the polymer obtained from commercial LA in 7 hours of polymerization, which indicates that the monomer obtained from fermentation has to be improved. Contaminants present in lactic acid can influence in the polymerization process, thus decreasing the yield and molecular weight (Orozco *et al.*, 2014).

Table 4-8 Results of polymer obtained from monomer produced by fermentation

Time (hours)	Yield (%)	Mv (g/mol)
4	55	1870
8	56	2370

4.3.1.3 Factorial experimental design for azeotropic dehydration of lactic acid

This study evaluated the occurrence polycondensation during azeotropic dehydration by the acid value method, which quantified the carboxylic groups in the LA molecule. In the polycondensation reaction, the carboxyl and hydroxyl groups are reactive with each other; the reduction of the amount of carboxyl groups indicates the occurrence of condensation with hydroxyl group (Jiménez-Bonilla *et al.*, 2014; Lucas *et al.*, 2001).

The acid value of LA is 0.584 g of KOH/g. According to

Table 4-9, in all essays, the acid value decreased, which indicates that, even with the water removal, the condensation of monomers occurs. The lowest acid value was 0.503 g of

KOH/g (86.1% of relative acid value), obtained using toluene/LA with a ratio of 3/1 and 6 hours of reaction, indicating that a higher ratio of toluene/LA and longer reaction time may decrease the acid value.

Table 4-9 Results of dehydration of lactic acid process

Essay	Toluene/LA ratio (v/v)	Time (hours)	Acid Value (g of KOH /g)	Relative Acid value (LA monomer - 100%)	Yield
1	1	2	0.555	95.0%	100%
2	1	4	0.521	89.2%	100%
3	1	6	0.518	88.7%	98%
4	2	2	0.516	88.4%	97%
5	2	4	0.507	86.8%	96%
6	2	6	0.505	86.5%	96%
7	3	2	0.512	87.7%	99%
8	3	4	0.510	87.3%	94%
9	3	6	0.503	86.1%	97%

Acid value of LA = 0.584 g of KOH /g

In table of estimated effects (Table 4-10) it is possible to verify that toluene/LA ratio has significant influence on acid value ($p<0.05$) and the variable time presented p-value of 0.0657, it has no significant influence at $p<0.05$, however, there is significant effect at $p<0.10$. A contour plot of acid value as function of toluene/LA and time (Figure 4-4) was generated, which is possible to observe that the region of lowest acid value obtained is using toluene/LA ratio higher than 2 and 4 hours of reaction.

Table 4-10 Estimated effect of azeotropic dehydration conditions on acid value results for. R^2=0.823.

	Effect	Std.Err.	t(4)	p
Mean/Interc.	0.884	0.005	166.714	$7.765x10^{-09}$
(1) Toluene/LA ratio (L)	-0.039	0.012	-3.027	0.038
Toluene/LA ratio (Q)	-0.017	0.011	-1.570	0.191
(2) Time (L)	-0.033	0.012	-2.514	0.065
Time (Q)	-0.009	0.011	-0.859	0.438

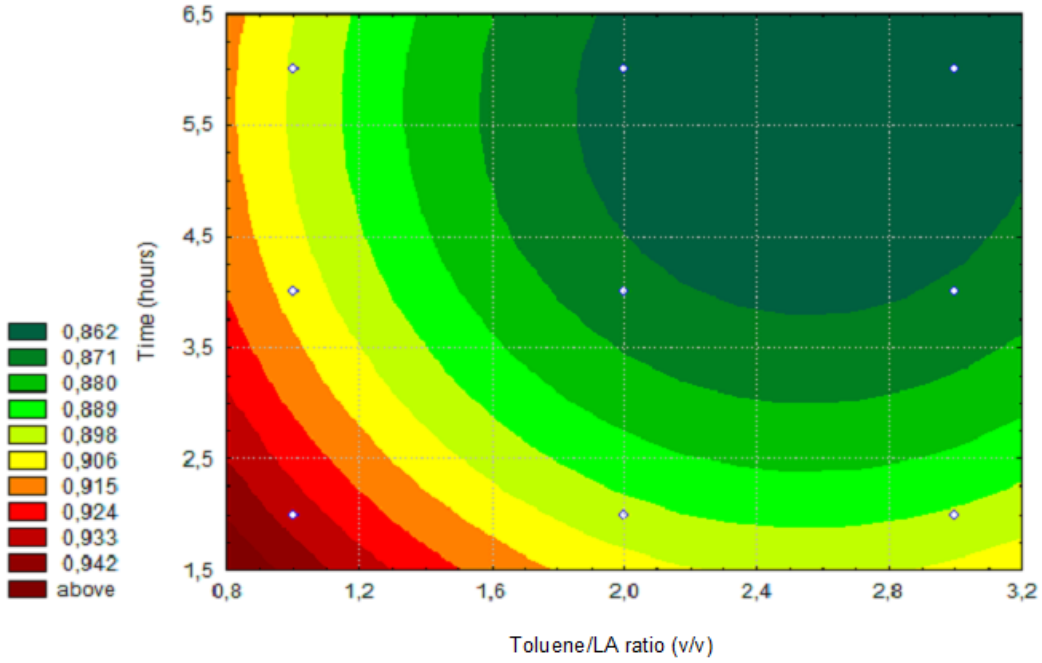

Figure 4-4 Contour plot of acid value for azeotropic dehydration of LA

4.3.2 PLA Synthesis in Microwave Oven

4.3.2.1 Polymerization of commercial lactic acid

The study, using a commercial LA, evaluated the influence of power, temperature and time on polymerization process. All samples were previously submitted to azeotropic dehydration using toluene as solvent in rotatory evaporator equipment at 90ºC. The experiment was conducted according to a Box-Benkhen experimental design and the dependent variables were the yield and viscosimetric molecular weight (Mv) (Table 4-11). The yield of polymerization varied from 40% (run 8) to 79% (run 1). At low power studied, 100 W, high yields were obtained, but the yield decreased with the increase of power. The color of polymer after the precipitation changed according to the power of the microwave and time of radiation. Low power and time resulted in a white color polymer and high power and time resulted in a brown color polymer.

The polymers obtained from this study are considered low molecular weight or oligomers. The highest molecular weight obtained was 2070 g/mol at run 2 (300 W, 140 ºC and 90 minutes).

Table 4-11 Results of Box-Behnken experimental design for polymerization of commercial lactic acid by microwave heating process

Run	Power	Temperature	Time	Color	Yield (%)	Mv (g/mol)
1	100	140	90	pale yellow	79%	1190
2	300	140	90	pale brown	45%	2070
3	100	180	90	pale yellow	76%	1670
4	300	180	90	pale brown	45%	1620
5	100	160	60	white	73%	1030
6	300	160	60	pale yellow	55%	1190
7	100	160	120	pale yellow	73%	1110
8	300	160	120	brown	40%	1220
9	200	140	60	white	64%	920
10	200	180	60	white	67%	1050
11	200	140	120	pale yellow	57%	1030
12	200	180	120	pale yellow	56%	1260
13	200	160	90	pale yellow	71%	1260
14	200	160	90	pale yellow	71%	1250
15	200	160	90	pale yellow	78%	1270

The obtaining of low molecular weight polymers was probably due to the influence of pressure. In this work, reactions in the microwave were subjected to ambient pressure. It is known from literature that the decrease in pressure favors the elimination of water formed during the polycondensation and, thus, increases the molecular weight of the polymer (Nakamura *et al.*, 2010). As the use of vacuum, nitrogen atmosphere (Chen *et al.*, 2006; Nagahata *et al.*, 2007) have been reported to promote the increase of the molecular weight.

The results of ANOVA (Table 4-12), according to the Box-Behnken design, demonstrate that power and time have significant p-value on the dependent variable (yield) ($p<0.05$). The effect of these independent variables on yield is negative, with the increase of time and power the yield decrease. This effect could occur due to degradation during polymerization. Kéki et al. (2001) studied polycondensation reaction at different times of radiation and observed that the yield decreased with increasing irradiation time. This was probably due to the loss of oligomers of lower mass during polycondensation. This result could be confirmed by the contour plot resulted (Figure 4-5). The region in dark color represents the highest yield of polymerization. This region is limited by time around 60 and 100 minutes and power less than 140-160 W.

Table 4-12 ANOVA of effect polymerization by microwave heating on yield. R^2=0.948

	SS	df	MS	F	p
(1) Power (L)	0.167	1	0.167	112.022	0.000006
Power (Q)	0.015	1	0.015	10.309	0.012
(2) Temperature (L)	0.000003	1	0.000003	0.002	0.963
Temperature (Q)	0.012	1	0.012	8.050	0.022
(3) Time (L)	0.013	1	0.013	9.205	0.016
Time (Q)	0.017	1	0.017	11.775	0.009
Error	0.011	8	0.001		
Total SS	0.232	14			

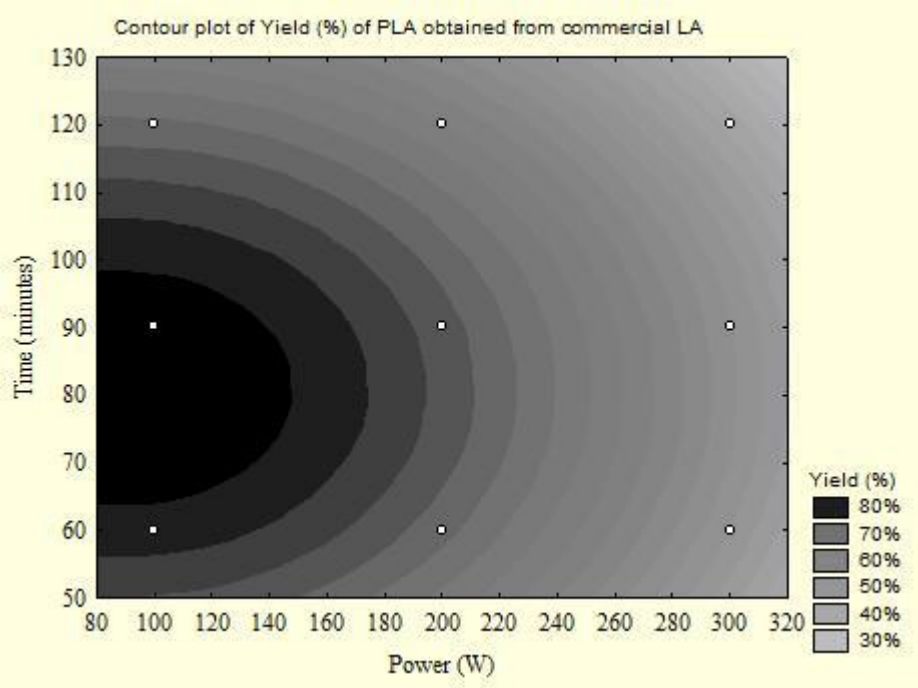

Figure 4-5 Contour plot of effect of time and power on yield of polymerization by microwave heating using a commercial lactic acid.

The independent variable temperature did not demonstrate significant p-value for the levels tested in this study. In the contour plot with time versus temperature and power versus temperature, the yield values are similar. Probably temperature could have a significant effect if the range of temperature studied was larger.

The ANOVA (Table 4-13), with the analysis of molecular weight, demonstrated that only the power and time influence the polymer molecular weight significantly ($p < 0.05$). The contour plot with power versus reaction time (Figure 4-6) shows that there is a tendency to increase the molecular weight by increasing the power of the microwave. However, low-power experiments combined with central levels of reaction time also resulted in high molecular

weights. The effect of various microwave power was studied by Yamada et al. (2014). At 100W of microwave power, the polycondensation did not occur. When increasing microwave power, the molecular weight increased and at 300W of power, a PLLA with higher Mn and Mw values was obtained (4400 and 11600, respectively).

Table 4-13 ANOVA of the effects of commercial lactic acid polymerization by microwave heating process on molecular weight R^2=0.777

	SS	df	MS	F	p-value
(1) Power (L)	337636.9	2	168818.5	4.806	0.042
(2) Temperature (L)	105133.8	2	52566.92	1.497	0.280
(3) Time (L)	476497.6	2	238248.8	6.783	0.019
Error	280973.7	8	35121.71		
Total SS	1260559	14			

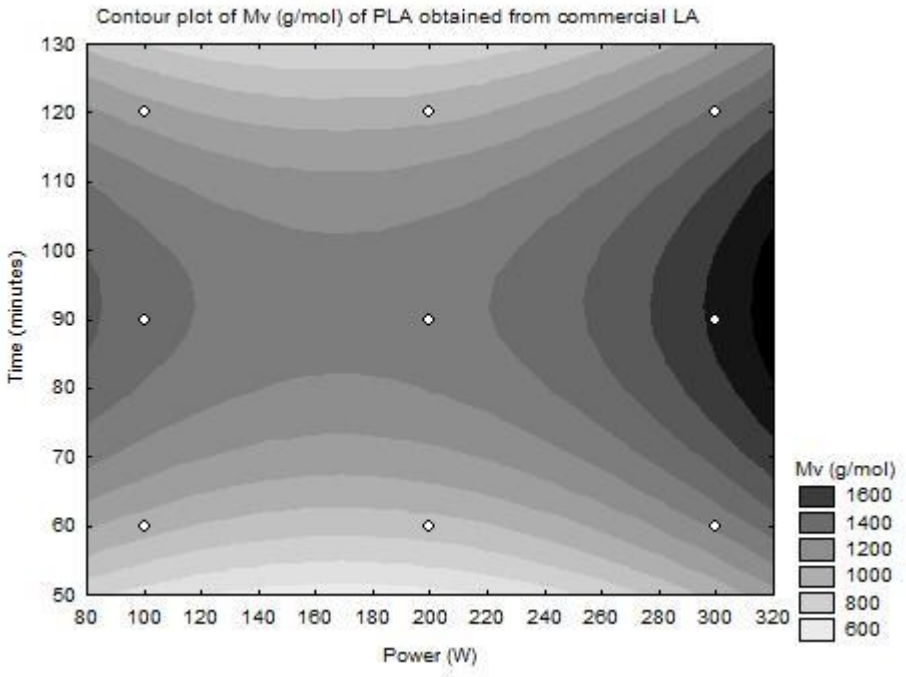

Figure 4-6 Contour plot of the effect of time of reaction and power on polymer's molecular weight obtained by microwave heating system.

The aim of this study was to define the condition that would favor a higher molecular weight and yield. It can be noted in Figure 4-7 that the highest yield (run 1) does not correspond to higher molecular weight (run 2). Characterization studies of PLA, was carried out with the polymer obtained in run 3, which originated the second largest molecular weight and yield.

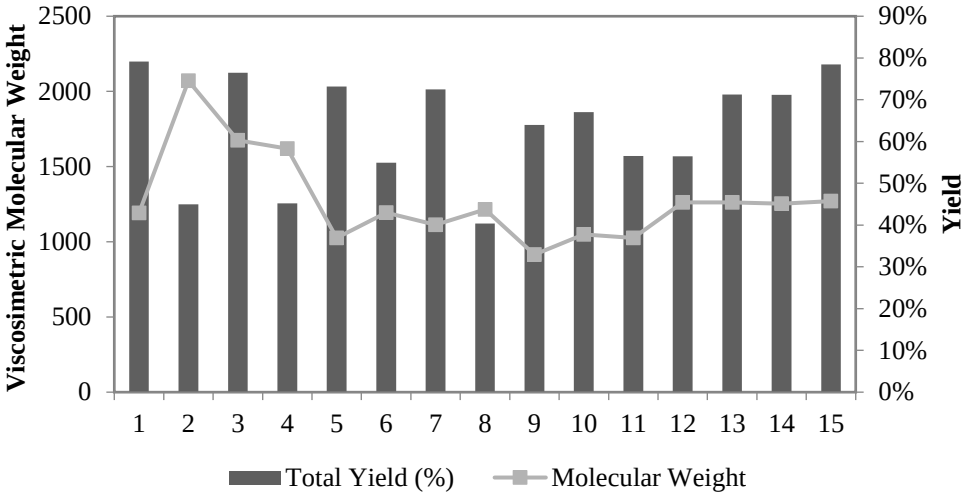

Figure 4-7 Molecular weight and yield results according to Box-Behnken experimental design for commercial lactic acid polymerization

4.3.2.2 PLA obtained from Lactic acid produced by fermentation

The polymerization of the lactic acid obtained by fermentation was conducted under the same conditions as the experiment conducted using commercial lactic acid. In this way, the main goal was to compare the polymer obtained. Commercial lactic acid had 100% of L-isomer and Fermented lactic acid had 95% of L-lactic acid and 5% of D-lactic acid. The tendency observed in the results of yield and molecular weight was similar in both experiments. According to Table 4-14, the highest yield obtained was 76% while the commercial lactic acid resulted in a polymer of 79% in the same test condition (100 W, 140 ° C and 90 mintes). The polymer with a higher molecular weight, 1450, was obtained in the same condition as the result obtained using commercial lactic acid (300W, 140 °C and 90 minutes).

Table 4-14 Results of polymerization by microwave of lactic acid obtained by fermentation

Run	Power	Temperature	Time	Color	Yield	Viscosimetric Molecular Weight
1	100	140	90	yellow	76%	610
2	300	140	90	pale brown	49%	1450
3	100	180	90	yellow	73%	840
4	300	180	90	pale brown	26%	930
5	100	160	60	yellow	64%	490
6	300	160	60	brown	46%	780
7	100	160	120	yellow	64%	480
8	300	160	120	brown	29%	550
9	200	140	60	yellow	54%	670
10	200	180	60	yellow	54%	850
11	200	140	120	yellow	39%	480
12	200	180	120	yellow	37%	650
13	200	160	90	yellow	59%	710
14	200	160	90	yellow	66%	700
15	200	160	90	yellow	62%	700

In this experiment, it was observed that, as in the previous experiment, the increase in power induced the polymer to a high a molecular weight and decreased the yield and lower power resulted in increased yield and decreased molecular weight. The results of ANOVA (Table 4-15 and Table 4-16) showed power and time had significantly influence, with $p<0.05$, on yield of polymerization and molecular weight of polymers. The contour plot generated (Figure 4-8), power versus time for molecular weight, indicates the influence of the increase of power on molecular weight and over than 300 W. It is possible to verify the optimal point for this dependent variable.

Table 4-15 ANOVA of effect of power, time and temperature for yield of polymerization of fermented lactic acid. R^2=0.897

	SS	df	MS	F	p-value
(1) Power (L)	0.1678	1	0.1678	112.0218	0.000006
Power (Q)	0.0154	1	0.0154	10.3091	0.0124
(2) Temperature (L)	0.000003	1	0.000003	0.0023	0.9633
Temperature (Q)	0.0120	1	0.0120	8.0508	0.0219
(3) Time (L)	0.0137	1	0.0137	9.2052	0.0162
Time (Q)	0.0176	1	0.0176	11.7751	0.0089
Error	0.0119	8	0.0014		
Total SS	0.2328	14			

Table 4-16 ANOVA results for molecular weigth of polymers obtained from fermented lactic acid. R^2=0.754

	SS	df	MS	F	p-value
(1) Power L	520085.4	2	260042.7	6.850	0.018
(2) Temperature L	95782.06	2	47891.03	1.261	0.334
(3) Time L	282464.8	2	141232.4	3.720	0.072
Error	303687.4	8	37960.93		
Total SS	1235026	14			

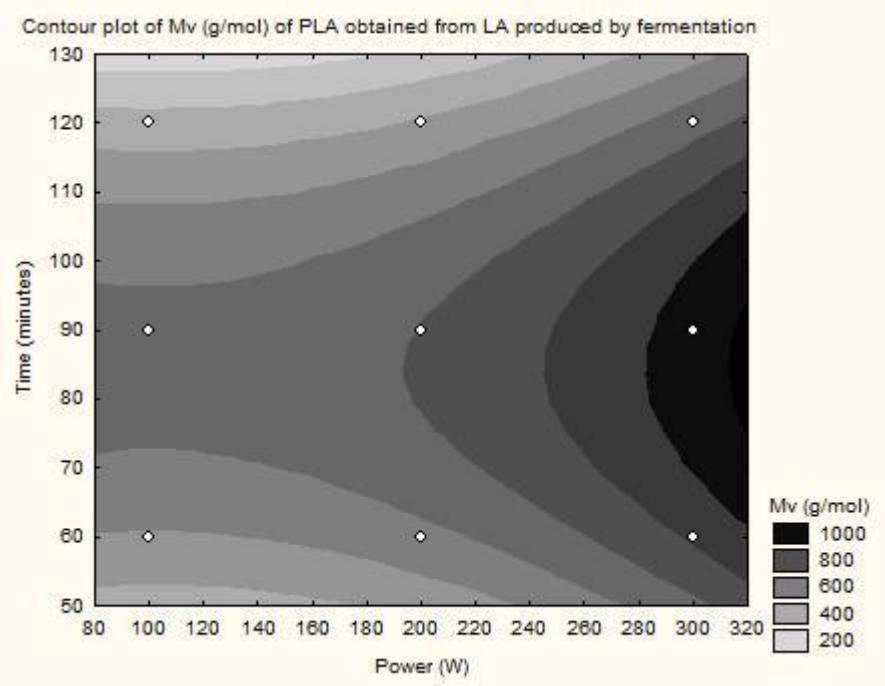

Figure 4-8 Contour plot of the effect of power and reaction time of polymerization on PLA molecular weight obtained from fermented lactic acid.

As in the previous study with commercial lactic acid, plotting the two results on a graph (Figure 4-9), run 1 provided the highest yield while run 2 provided the highest molecular weight. The polymer obtained in run 3 was chosen for characterization studies.

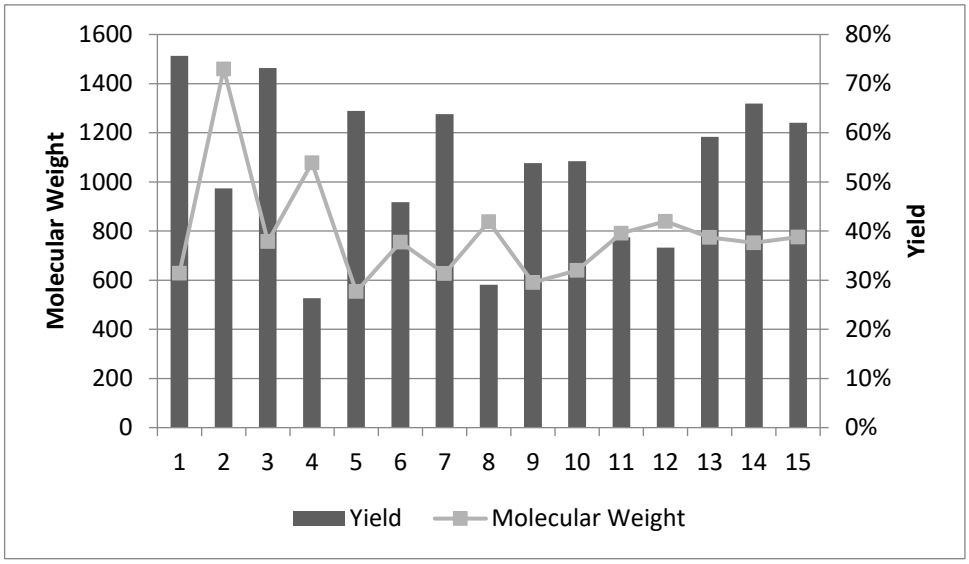

Figure 4-9 Molecular weight and yield results according to Box-Behnken experimental design for fermented lactic acid polymerization

4.3.3 Conventional heating system versus microwave heating system

Both heating systems provide the polymerization of lactic acid (commercial and fermented). The microwave reached higher yields, 79%, however low molecular weight, around 2000, were reached. This fact was due to the environment pressure that did not facilitate water removal. The final aspect of the precipitated polymer was different. In conventional heating polymerization, the polymer was obtained in powdered form, while the polymer from microwave polymerization, was obtained as a gel form (Figure 4-10). Low molecular weight contributes to the gel aspect of the sample.

The consumed energy demand is different in the two systems and can be calculated by the equation 4-6. The data in Table 4-17 represent the energy of each process. The microwave spends about 20 times less energy than the conventional process.

$$E = \frac{P \times t}{1000} \quad (4\text{-}6)$$

Where: E: electric energy consumed (kJ); P: power (W); t: time (s)

Table 4-17 Energy consumption estimation during polymerization process by conventional and microwave heating system

Conventional heating process	Microwave heating process
W= 25 g	W = 15 g
P = 500 W	P = 100 W
t = 10 h (36,000 s)	t = 90 minutes (5400 s)
E = 18,000 kJ	E = 540 kJ
E/W = 720 kJ/g	**E/W = 36 kJ/g**

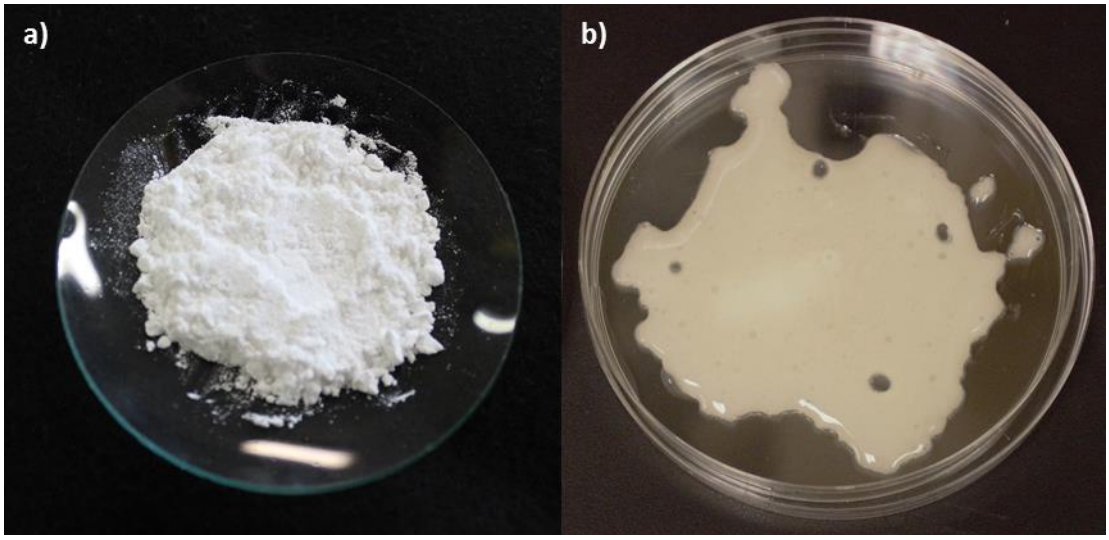

Figure 4-10 PLA obtained by different polymerization heating system. A) Powered form obtained by Conventional heating; b) Gel form obtained by Microwave heating.

4.4 CONCLUSIONS

Zinc lactate is a good alternative to $Sn(Oct)_2$ commonly used as catalyst in polymerization of lactic acid by polycondensation and ring opening polymerization.

Conventional heating process provided polymers with higher molecular weight than microwave heating system but lower yield. A polymer with 6334 g/mol of molecular weight and 61% of yield was obtained from commercial lactic acid after 2 hours of azeotropic dehydration, 1.5 toluene/LA ratio, 10 hours of polycondensation and 0.0006 catalyst/LA ratio. Microwave heating process provided a polymer with 2071 g/mol under 300 W, 140 ºC and 90 minutes.

The polymerization time is very important and in this study, it was used 10 hours of polymerization for conventional heating process and 90 minutes for microwave heating, which is below than the time described in the literature for polycondensation reaction.

The developed polymerization process led to polymers in powder form (conventional process) and polymer in gel form (microwave process). Low molecular weight influences in the form of gel polymers. The energy required for polymerization using microwave is lower than using conventional heating process. This is one of the advantages of microwave, another advantage is the short time of reaction (90 minutes).

CHAPTER 5 – CHARACTERIZATION STUDIES OF POLY(LACTIC ACID)

ABSTRACT

This chapter aimed the characterization of PLA polymers obtained by different polymerization methods, conventional and microwave heating system using as monomers commercial LA and LA produced by *Lactobacillus pentosus*. Physico-chemical properties were determined such as the viscosimetric molecular weight, morphology by SEM, degree of crystallinity by DXR, functional groups by FTIR, thermal properties (Tg, Tc, Tm) by DSC and thermal stability by TGA. The biopolymers obtained by conventional heating polymerization (both from commercial monomer and produced monomer) were presented in solid form with a degree of crystallinity varying according to the reaction time. The polymer obtained from the monomer produced by fermentation and also 8 hours of reaction present 31% of crystallinity. Thus, it is possible to confirm that the reaction time strongly influenced the crystallinity and molecular weight, as well as the thermal transition temperatures and thermal stability. The polymers obtained by microwave polymerization presented a gel consistency at room temperature because of its low molecular weight, and all polymers had Tgs below 0ºC and low thermal stabilities. Comparing conventional and microwave heating method, better results were obtained, in terms of physico-chemical properties, by the conventional heating method for application in encapsulation of bioactive compounds and tissue engineering.

KEYWORDS: Differential scanning calorimetry (DSC), Fourier Transform Infrared (FTIR), X-ray Diffraction (XRD), Thermogravimetric Analysis (TGA) and Scanning Electron Microscopy (SEM).

5.1 INTRODUCTION

Biomaterials have shown a highly exploitable area, since a very large variety of biopolymers can be obtained from renewable sources. They present advantages, in comparison to petroleum based non-renewable polymers, which are very attractive and help the reduction of the petroleum polymers use and environmental impact (Madhavan Nampoothiri *et al.*, 2010).

Biopolymers present a wide range of applications, from packaging till biomedical applications. For a given application, there are requirements concerning the knowledge of the specific physicochemical properties of the biopolymer. Thus, one of the most important is the biodegradability, which depends on the molecular weight, molecular form and crystallinity (Premraj and Doble, 2005).

Poly(lactic acid) (PLA) is a biopolymer, which has been studied for different applications, depending on its chemical nature., It presents three forms: PLLA, PDLLA and PDLA, because the monomer presents two optical isomers, L and D. The stereochemistry, the ratio of L and D isomers, influences on PLA properties and degradability (Abdel-Rahman et al., 2011).

PLAs with L-content (PLLA) greater than 90% tend to be crystalline while those with lower optical purity are amorphous. The melting temperature (Tm), glass transition temperature (Tg), and crystallinity decrease with decreasing amounts of L-isomer (Lasprilla et al., 2012). Pure PLLA and PDLA have properties such as Tg between 50 and 70ºC, Tm between 170 and 190ºC, and a crystallinity of around 35% (Fambri and Migliaresi, 2010). Physical characteristics depend on the transition temperatures, such as mechanical and rheological characteristics, density and heat capacity (Henton *et al.*, 2005; Lim *et al.*, 2008). PLA has lower Tm and Tg than polyethylene terephthalate (PET) and polystyrene (PS), which make PLA better for heat sealing and thermal processing.

Moreover, PLA properties (crystallinity and thermal properties) are influenced by the polymer molecular weight, polymerization conditions, thermal history and purity (Fambri and Migliaresi, 2010).

This chapter aims to present the physico-chemical properties' characterization of different PLA obtained from LA monomer polymerization fermentation focusing the use of these polymers in the encapsulation of bioactive compounds.

5.2 MATERIAL AND METHODS

5.2.1 Material

Poly (lactic acid) standard (Mn=30,000 g/mol and Mw=60,000 g/mol) was obtained from Sigma Aldrich®, USA.

PLA obtained by different polymerization methods (conventional and microwave heating) according to Chapter 4.

5.2.2 Polymers

Monomers of lactic acid, lactic acid commercial (L-isomer) (VETEC, Brazil) and lactic acid produced by fermentation (95% L-isomer and 5% D-isomer), were submitted to different

polymerization methods: conventional and microwave heating process as described in Chapter 4. For characterization studies, it was choosen the polymers according to Table 5-1.

Table 5-1 Different PLA samples used in characterization studies

Polymer	Source	Polymerization type	Mv (g/mol)	Yield of polymerization (%)
PLACC	Commercial LA	Conventional	6330	61
PLAFC	LA produced by fermentation	Conventional	2370	56
PLACMW	Commercial LA	Microwave	2070	45
PLAFMW	LA produced by fermentation	Microwave	1450	49

PLACC – Poly(lactic acid) from commercial monomer and conventional polymerization; PLAFC – Poly(lactic acid) from fermented monomer and conventional polymerization; PLACMW - Poly(lactic acid) from commercial monomer and microwave polymerization; PLAFMW - Poly(lactic acid) from fermented monomer and microwave polymerization

5.2.3 Morphology by scanning electronic microscopy SEM

The morphology of PLA pellets was examined by scanning electronic microscopy (SEM) Teskan mod Vega 3LMO (Oxford Instruments), operating at 15 keV. Before examination, the samples were sputter-coated with a thin layer of gold in a vacuum chamber.

5.2.4 PLA thermal stability by thermogravimetry (TG)

Thermal stability of biopolymers was evaluated by thermogravimetric analysis in a SETARAM (Setsys Evolution TG/DTA/DSC), under argon atmosphere from 20ºC to 600ºC at a heating rate of 20ºC/min and under oxygen atmosphere up to 800ºC at 20ºC/min and gas flow of 20 mL/min.

5.2.5 Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) measurements were performed in a DSC NETSCHZ thermal analyzer under nitrogen atmosphere. A weighted sample of more than 12 mg was collected in an aluminum pan (Al-Crucibles 20 μL) and completely sealed. The measurements were carried out from -30 to 200 °C at a heating rate of 10 °C/min and at a cooling rate of 10°C/min. To eliminate the thermal history, all the samples were heated up to 200°C, and then cooled to -30 ºC. The glass-transition temperature (*T*g) and the crystallinity data were recorded from the second heating curve, from -30 ºC to 200 ºC at a heating rate of 10 ºC/min.

Crystallinity values for different biopolymers were calculated through the integration of the normalized area of the melting endotherm, the determination of the heat involved, and the

rating of it to the reference 100% crystalline polymer (93.6 J/g) (Zhai *et al.*, 2009). Degree crystallinity was of all the samples were calculated using Equation 5.1 (Zafar *et al.*, 2016).

$$X = \frac{\Delta Hm - \Delta Hc}{\Delta H_m^0} \times 100 \quad (5.1)$$

ΔHm the heat of melting,

ΔHc the heat of cold crystallization,

ΔHm° heat of melting of 100% crystalline PLA samples (considered as ΔHm° = 93 J/g).

5.2.6 Degree of cristalinity by X ray diffraction (DRX)

Wide-angle X-ray scattering patterns of the samples were obtained in the reflection mode with a SHIMADZU XRD 700 MAXIMA diffractometer and Ni-filtered copper radiation (CuKα, λ = 1,5418 Å). The samples were scanned in the 2° range of 10–40 °, and the generator was operated at 40 kV and 20 mA. The full width at half-maximum of the 110 peak was determined with peak-fitting software available with the diffractometer.

5.2.7 Fourier transform infrared spectroscopy (FTIR)

The monomers, oligomers and polymers obtained from different synthesis were characterized by FTIR spectroscopy in a VERTEX 70 equipment (Bruker), with the accessory DRIFTS (diffuse reflectance) with 64 scans, 4 cm^{-1} resolution, without the elimination of atmospheric compensation. The samples were previously dried at temperature 100°C for 24 hours, then were ground and mixed to homogeneity in spectroscopic KBr and placed in DRIFTS accessories for the acquisition of the spectra.

5.3 RESULTS AND DISCUSSION

5.3.1 Morphology analysis of the biopolymers by scanning electronic microscopy (SEM)

Biopolymer morphology is shown in photomicrographs. Only powered polymers could be analyzed. The polymers in gel form (obtained by microwave heating polymerization) could not be analyzed by SEM due to the preparation step, which needs a solid sample for analysis.

The morphology of the polymer obtained from commercial LA (PLACC) (Figure 5-1) presents a globular aspect with inhomogeneous distribution in relation to the size of the globules

(Figure 5-1 a and b). In Figure 5-1 c it is possible to observe pulverulent appearance surfaces of the globule.

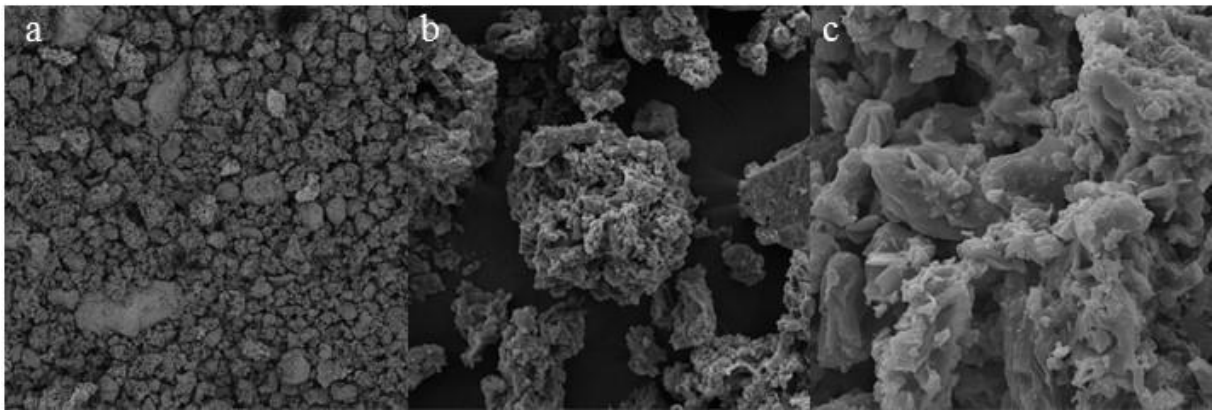

Figure 5-1 SEM micrograph of PLA obtained from commercial LA after 10 hours of polymerization. (a) 100x, (b) 1,000x and (c) 5,000x

The polymer obtained from LA monomers produced by *Lactobacillus pentosus* (fermentation conditions) after 8 hours of polymerization (PLAFC) (Figure 5-2), presents globules with higher porosity, which is a characteristic for application in drug system delivery (Fu *et al.*, 2002).

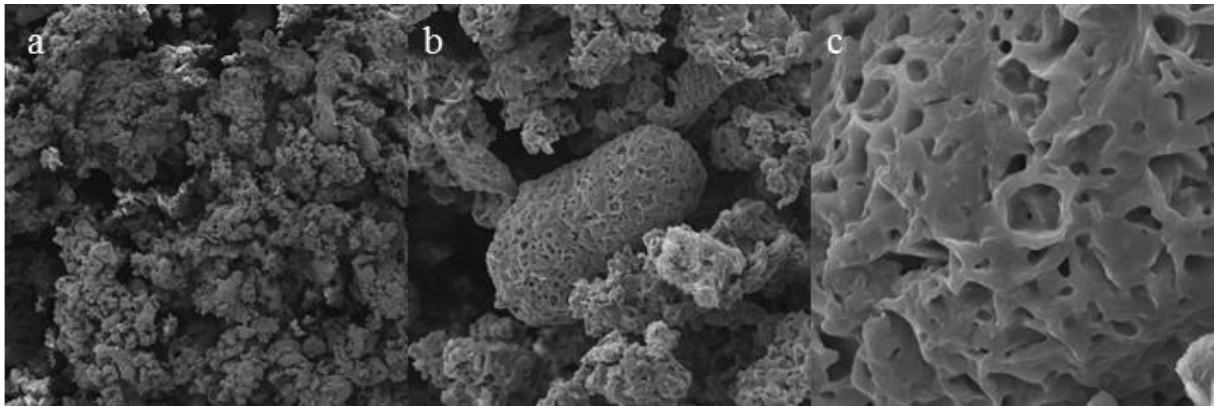

Figure 5-2 SEM micrograph of PLA obtained from LA produced by *Lactobacillus pentosus* after 8 hours of polymerization. (a) 100x, (b) 1,000x and (c) 5,000x

The morphology of commercial PLA (Figure 5-3) presents a granulated form, with dense and compact characteristics, striated surface (Figure 5-3 - b) and fibrillar structures.

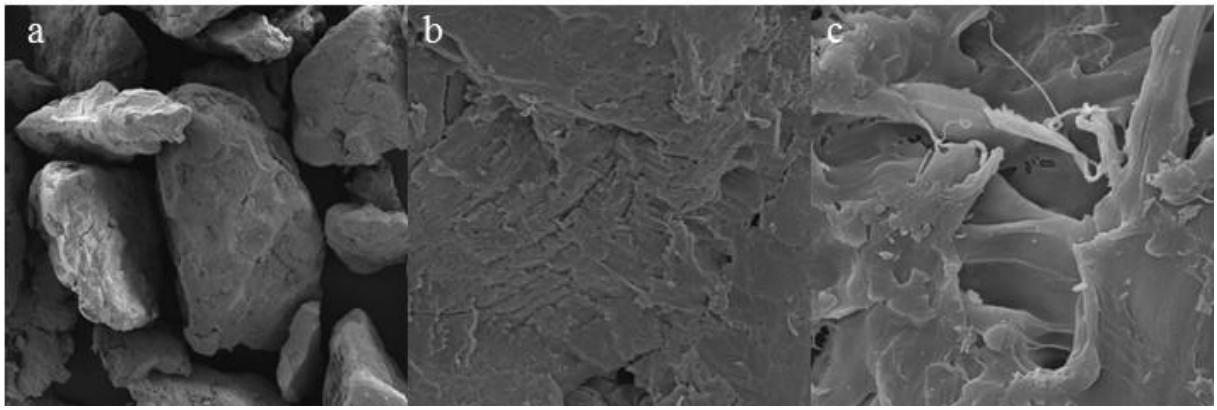

Figure 5-3 SEM micrograph of standard PLA. (a) 100x, (b) 1,000x and (c) 5,000x

The tendency of the globular morphology of the obtained polymer is a positive aspect for the application in encapsulation of bioactive as noted by Fu et al. (2002).

5.3.2 PLA Thermal stability by thermogravimetry (TG)

According to the results shown in Table 5-2 and Figure 5-4, it appears that the polymers have a similar thermal degradation profile with two distinct events, the first event up to 120-130°C due to the loss of adsorbed water or volatile compounds at this temperature. Each polymer exhibited weight loss at different temperatures, the second event between 175-380ºC is due to the thermal decomposition of the PLA polymers' chains (Petinakis *et al.*, 2010).

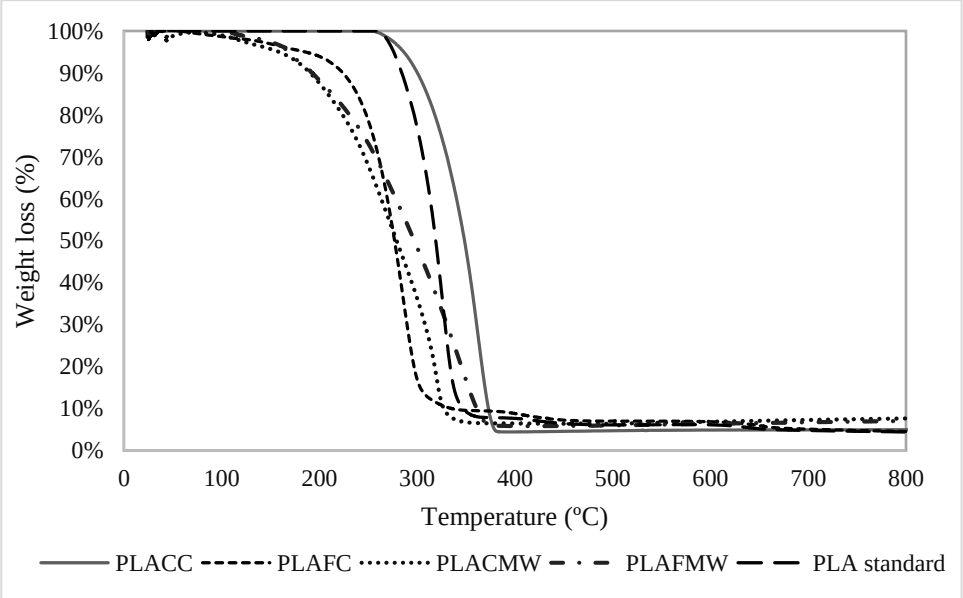

Figure 5-4 Thermal decomposition of PLA evaluated by TGA

The polymers obtained from conventional heating system have higher thermal stability than polymers obtained from microwave heating system. PLACC presented a thermal stability that was close to the PLA standard, up to 260ºC while PLAFC presented lower thermal stability (up to 200 ºC). This indicates that the decomposition of PLA obtained from produced LA starts earlier than that from PLA obtained from commercial monomer.

For the results of the thermal stability of PLA obtained from microwave heating system, two events appeared: the first at 130-140°C, which corresponds to water and volatiles compounds loss; the second event ranges from 130°C to 370°C. Both PLACMW and PLAFMW showed initial temperature of mass loss around 130-140°C, these polymers have good thermal stability up to 130°C.

The standard PLA presents good thermal stability up to 260ºC and total degradation at 360ºC and after this temperature, all organic material has been degraded. What remains are the inorganic compounds (ashes).

Table 5-2 Weight loss obtained from thermogravimetric curves for PLA

Polymer	Temperature (°C)	Weight loss (%)
PLACC	260-380	94.8
	800	4.7
PLAFC	130	2.2
	200-310	81.0
	800	4.5
PLACMW	140	6.7
	140-340	86.1
	800	7.3
PLAFMW	130	1.5
	130-370	91.9
	800	6.6
PLA standard	260-360	91.9
	800	4.4

5.3.3Differential scanning calorimetry (DSC)

Figure 5-5 and Table 5-3 present the results of DSC for PLA polymers. The polymers obtained from conventional heating polymerization presented glass transition temperature (Tg) higher than polymers obtained from microwave heating polymerization. Between polymers PLACC (Mv=6,330 g/mol) and PLAFC (Mv=2,370 g/mol) there is a significant difference in the Tg value, 40.8ºC and 18ºC, respectively. This difference occurs probably due to the presence

of some contaminants in the LA monomer produced by fermentation (PLFC), which affects polymerization reactions and, consequently, the properties of the polymers.

Differently from other polymers, only the PLACC presented crystallization peak (Tc) (95.6 ºC) and two melting peaks (Tm) (122.9 ºC and 133.9 ºC). This fact is reported in the literature. Tabi et al. (2010) reports that during the crystallization of PLLA, between 133-140ºC, there is an existence of a crystalline disorder α' which is produced by annealing at low temperature, transforming to a α ordered phase. The first Tm peak corresponds to α' and the second Tm peak corresponds to α, and this is a characteristic behavior of a low molecular weight PLA. Pereira and Morales (2014) and Di Lorenzo et al. (2011) observed that transition α' to α are dependent on the heating rate. It is explained by the occurrence of competitive effect fusion and recrystallization during the heating process. Imperfect and small crystals successively move to more stable crystals through this fusion-recrystallization mechanism. The endothermic peak would be shown when the melting rate exceeds the recrystallization and an exothermic signal when the recrystallization rate is higher than the fusion. As each process has a different kinetics depending on the heating conditions either be favored (Yasuniwa *et al.*, 2004).

Standard PLA showed Tg (60.8°C) and Tm (178.1 °C) values higher than PLACC polymer. Furthermore, it only showed one peak of Tm. The occurrence of only one peak can be related to the high molecular weight of this polymer, 30,000 g/mol.

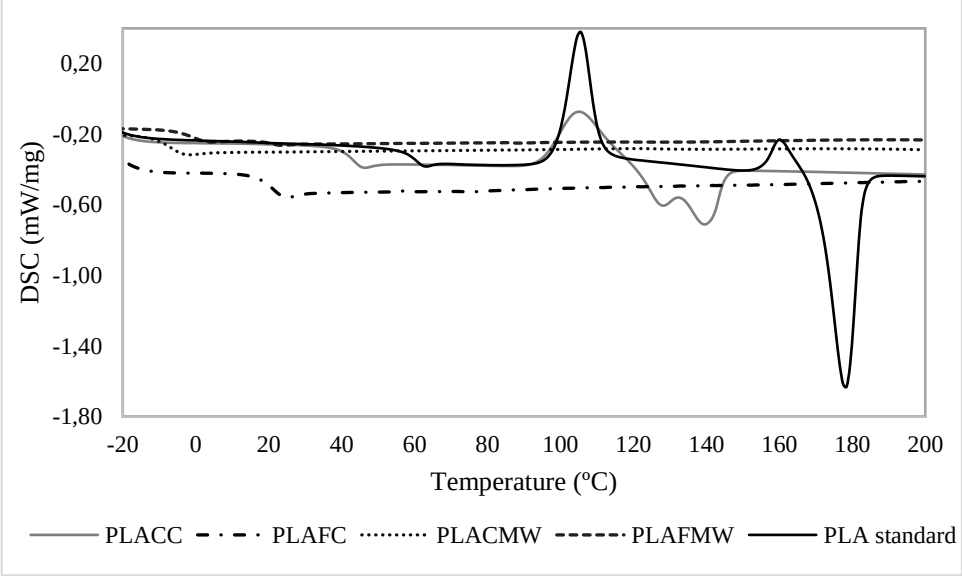

Figure 5-5 DSC curves for PLA obtained from conventional and microwave heating system method and standard PLA

Table 5-3 Thermal characteristics of PLA evaluated by DSC

	Tg (ºC)	Tc (ºC)	Tm (ºC)	Cristallinity (%)	ΔH (J/g)	Cp (J/g.K)	Mv (g/mol)
PLACC	40.8	95.6	122.4/133.9	11.00	-4.926/-9.295	0.422	6,330
PLAFC	18.0	-	-	-	-	0.598	2,370
PLACMW	-7.1	-	-	-	-	0.572	2,070
PLAFMW	-0.8	-	-	-	-	0.330	1,450
PLA Standard	60.8	105.3	178.1	22.2	-56.94	0.468	30,000*

*Molecular weight corresponds to Mn.

The results of DSC for polymers obtained from microwave heating polymerization (PLACMW and PLAFMW) showed the polymers did not present melting temperature (Tm), only Tg, whose values are below 0ºC. It was expected that the Tg was lower for polymers obtained by microwave than polymers obtained by conventional polymerization methods due to their molecular weight. According to Fambri and Migliaresi (2010), molecular weight, polymerization conditions, thermal history and purity influence the crystallization, crystallinity degree and thermal properties of PLLA.

5.3.4 Degree of cristalinity By X Ray diffraction(DRX)

Figure 5-6 shows XRD patterns of polymers and standard PLA, in which it is observed that all samples have the same diffraction profile, with small displacements in the peaks in the characteristic angles, as shown in Table 5-6. Santos and Tavares (2013) observed diffraction peaks of PLA at 16.9° and 19.0°. Tabi et al. (2010) observed diffraction peaks at 14.8°, 16.3°,19.0° and 22.3°, corresponding to the diffraction peaks found in the PLA obtained in this study. All samples exhibit semicrystalline material behavior as reported by Shyamroy et al. (2005) and Zhang et al. (2005).

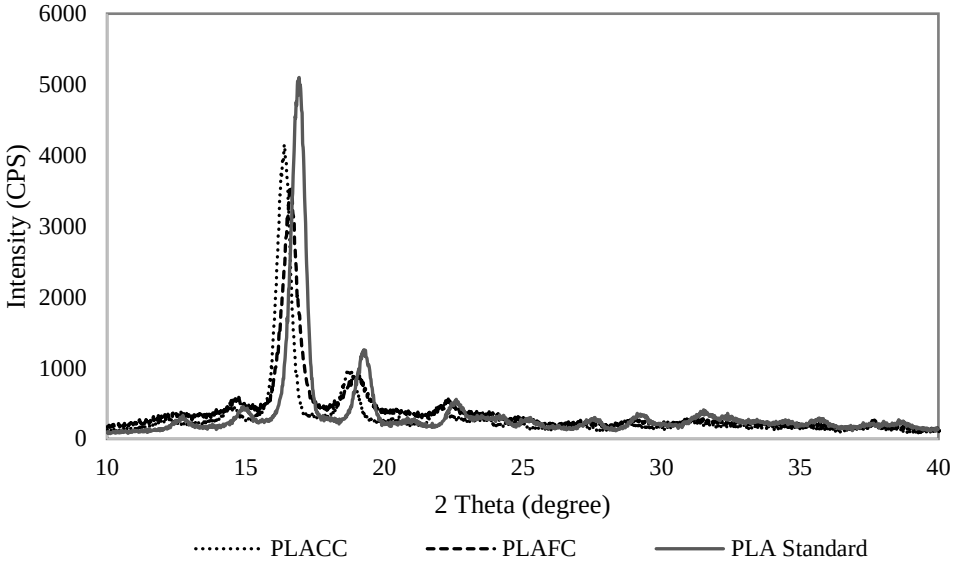

Figure 5-6 X-ray diffraction pattern of PLA standard and PLA obtained from different monomer source by conventional heating polymerization

From the curves of X-ray diffraction, the crystallinity of the biopolymers was calculated (Table 5-6). Crystallinity content of standard PLA is 48%, whereas the PLA obtained in this study have lower crystallinity. The crystallinity content of the polymers obtained with commercial LA was higher than the polymers obtained for fermented LA, thus PLACC has higher crystallinity, 36%, which was an expected result according to the data obtained in the DSC analysis, in which it was the only polymer that presented Tc and Tm. PLAFC has 31% crystallinity and presented no Tc and Tm the DSC results. Santos and Tavares (2013) obtained values of PLA pellets of 30% crystallinity.

Table 5-4 Diffraction peaks at 2θ and planes of PLA

	PLACC	PLAFC	PLA Standard
2θ/hkl			12.6
	14.4 (010)	15.2 (010)	14.9 (010)
	16.4 (200)/(110)	17.2 (200)/(110)	16.9 (200)/(110)
	18.7(203)	19.5 (203)	19.2 (203)
	21.9 (015)	22.7 (015)	22.6 (015)
% Cristallinity	36	31	48

5.3.5 Fourier transform infrared spectroscopy (FTIR)

Figure 5-7 show the infrared spectra of the monomer LA, standard PLA and PLA obtained by conventional heating polymerization. The polymers showed the same absorption

bands. A band around 3500 cm^{-1} represents OH group which corresponds to the water (Motta and Duek, 2006). This strong band in the monomer decreases and becomes more defined according to the polymerization time, thus indicating the higher molar mass of the polymer. All polymers showed absorption at 1750 cm^{-1}, C=O, however the absorption is lower in standard PLA compared to other polymers, Other absorption regions were observed at 1475 cm^{-1}, 1390 cm^{-1}, 1200 cm^{-1} and 1100 cm^{-1}.

According to Hu et al. (2012), the band at 956 cm^{-1} corresponds to the amorphous phase, whereas the band around 920 cm^{-1} is related to the ordered helical chain conformation in various crystal modification.

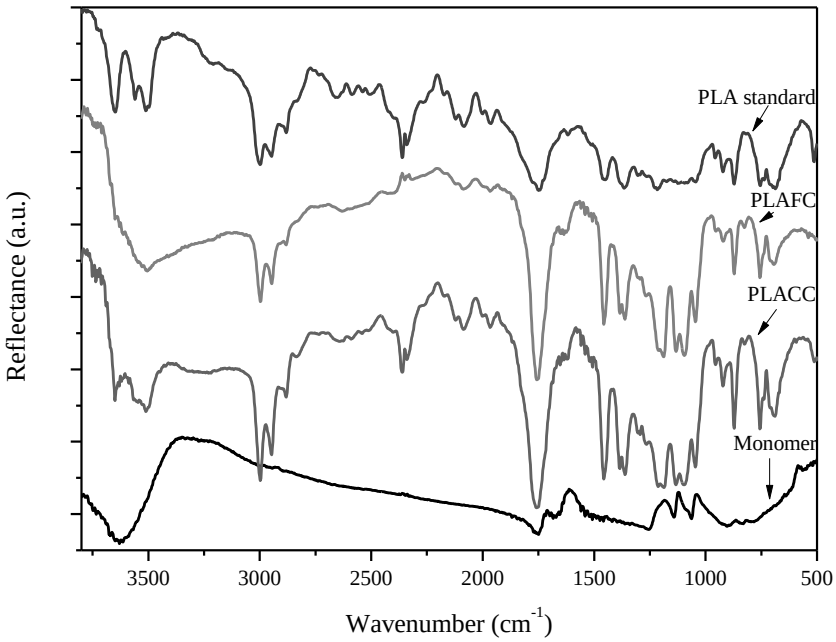

Figure 5-7 FTIR spectra of PLA obtained by conventional heating system polymerization.

In Table 5-5 it is possible to see the values of the bands of functional groups of the monomer and the polymers indicating the formation of the polymer. Orozco et al. (2014) also noted the presence of these bands. Similar regions were also reported by Kodama et al. (2009) and Krikorian and Pochan (2005).

The main differences between the spectra of the standard PLA and PLACC are in the region between 3600-2800 cm^{-1}. These are characteristics of the areas represented by the semi-crystalline and amorphous regions of these materials that result in deviations of the asymmetric and symmetric stretching vibration modes of the CH_3 groups. The region between 3600-3200 cm^{-1} is also attributed to the OH stretching vibration, the second region between 1800-1700 cm^{-}

[1], which is represented by the stretching vibration of C=O group and angular deformation of the CH_3 groups (1400-1300 cm^{-1}). Similar behavior was reported by Zhang et al. (2005), which attributed the phenomenon to phase transition during the crystallization of the form α '/α. HU et al. (2012) also reports the same phenomenon, though still adds that other regions to the same transition α'/α between 871 cm^{-1} (-C-COO stretching vibration) and 757 cm^{-1} assigned to angular deformation of the C=O group.

The band around 3200 cm-1 is related to the stretching of OH group. This decreases from the monomer to the polymer due to the polyesterification reaction that consumes the OH groups when they react with the acid groups to form the ester (Motta and Duek, 2006).

Lasprilla et al. (2011) observed a band shift related to the C=O stretch in the monomer in 1,727.06 to 1,757.92 cm^{-1} in the polymer. The difference in the peak intensity suggests the arrangement of molecules in the polymer chain.

Table 5-5 FTIR spectra – Bands assignments of PLA obtained by conventional heating system

Monomer	PLA Standard	PLACC	PLAFC	Assignments
3633	3650; 3561; 3494	3649; 3629	3514	O–H stretching vibrations
-	3003; 2943	3001	3004	CH_3 symetric vibrations stretching
-	2877	2949; 2883; 2839	2952; 2885	CH_3 and CH_2 vibration symmetric
1760	1755	1766	1753	C=O vibration stretching
1674	1620; 1616	1652; 1649	1623	OH deformation angle
-	-	1577	-	CH_3 bending vibrations asymmetric
-	-	1540	-	C-H Bendig vibratio
-	1469	1458	1456	Splitting of the CH3 asymmetric deformation
-	1444	-	-	CH_3 bending vibrations symmetric
-	1369	1386	1386	CH_3 asymmetric torsion
-	-	1357	1359	C–O asymmetric stretching vibrations
1263	1218	1294	1263	O–H deformation
1147	1112	1188	1217	C-O deformation
-	1085	1136	1186	O-C-C Vibration
1066	-	1093	1097	C-O Assymmetric vibration
-	1043	-	1049	C-C= Stretching vibration
-	964	958	956	Coupling of the C-C backbone stretching with mode Rocking deformation CH_3
-	927	925	918	C-H
889; 800	875; 779	873; 757	867; 831; 761	CH_3 asymmetric torsion
-	694	688	694	O–H stretching vibrations

Figure 5-8 shows the FTIR spectra of PLA polymers synthesized by microwave. It is observed that all the samples have the same characteristic bands of these materials. This behavior is expected since the reaction conditions were well controlled. It evidenced the fact that the reaction system is reproducible, since the formed products have the same spectrum as in Table 5-3, which also shows the bands and their assignments, although with small shifts in some bands, but still is within the assessed resolution (4 cm^{-1}).

This similarity observed in all FTIR spectrum can be attributed to the fact that the samples are in gel form (large contribution of amorphous domains) without preferential orientation chains, which does not show PLA polymorphism.

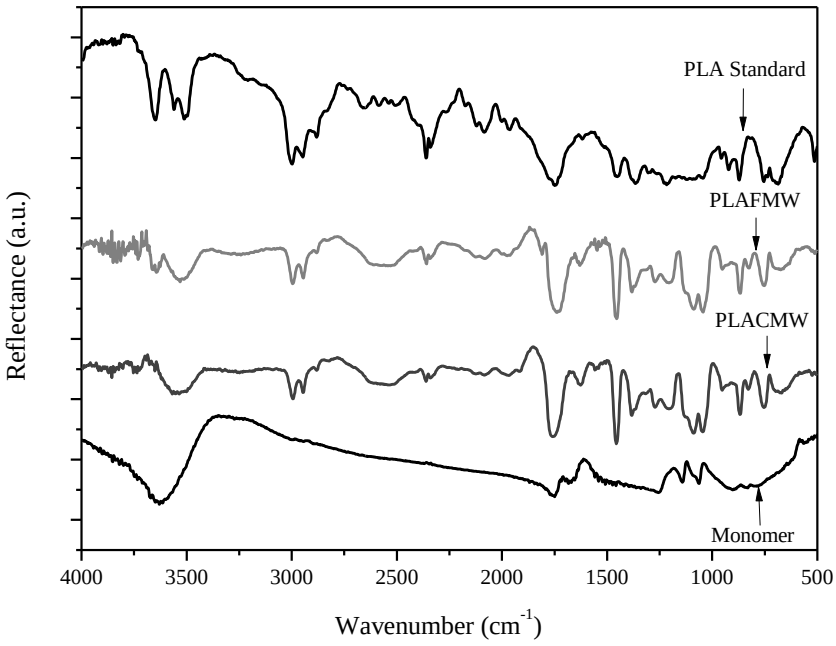

Figure 5-8 FTIR spectra of PLA obtained by microwave heating polymerization

Table 5-3 FTIR spectra of PLA – Bands assignments of PLA obtained by microwave heating

Monomer	PLA Standard	PLAFMW	PLACMW	Assignments
3633	3650; 3561; 3494	3650; 3504	3496	O–H stretching vibrations
-	3003; 2943	2995; 2947	2991; 2949	CH_3 symetric vibrations stretching
-	2877	2887	2883	CH_3 and CH_2 vibration symmetric
1760	1755	1759	1751	C=O vibration stretching
1674	1620; 1616	1629	1637	OH deformation angle
-	-	-	-	CH_3 bending vibrations asymmetric
-	-	-	-	C-H Bendig vibratio
-	1469	-	-	Splitting of the CH3 asymmetric deformation
-	1444	-	-	CH_3 bending vibrations symmetric
-	1369	1386; 1363	1386; 1369	CH_3 asymmetric torsion
-	-	1274	1276	C–O asymmetric stretching vibrations
1263	1218	1195	1197	O–H deformation
1147	1112; 1085	1136; 1097	1130; 1095	C-O deformation
1066	-	-	-	O-C-C Vibration
-	1043	1051	1045	C-O Assymmetric vibration
-	964	956	956	C-C= Stretching vibration
-	927	920	920	Coupling of the C-C backbone stretching with mode Rocking deformation CH_3
889; 800	875; 779	831; -757	829; 757	C-H
-	694	669	669	CH_3 asymmetric torsion

FTIR analysis for all produced PLA confirms that there was the formation of polymers comparing the spectra of the monomer, polymer (standard) and the produced PLA presented in this thesis. However, due to racemic mix of monomer, the reaction of polymerization occurs with formation of PLA-DL, which can be evidenced by characteristics bands of FTIR and transition temperatures of polymer.

5.4 CONCLUSION

The polymers obtained in this work from monomers produced by fermentation and conventional heating polymerization presented physicochemical properties consistent with reported in the literature, demonstrating the possibility of the use of these materials in applications that requires high porosity, tendency to sphere formation and do not require high molecular weight for encapsulation of bioactive compounds and tissue engineering.

Moreover, the polymers obtained by microwave heating polymerization, spite of its low weight and lower the Tg, exhibited similar infrared spectra, thus polymerization process is homogeneous and reproducible. These polymers form film high supported.

CONCLUSIONS

In this work the production of lactic acid by fermentation was developed using potato processing waste (HPPW) and sugarcane juice (SCJ) the substrate. The strain of *L. pentosus* has been adapted to a medium based on a by-product of the agroindustrial low value instead of synthetic medium. In both substrates lactic acid bacteria *L. pentosus* was able to produce high concentrations of LA, 150 g/l using HPPW and 225 g/l using SCJ. The HPPW requires an acid pre-treatment under high temperature to hydrolyze the starch to glucose and maltose. Contrarily, sugarcane juice does not require pre-treatment.

The use of baker's yeast as a source of nitrogen is another alternative reduction of LA production costs. The fermentation in nonsterile conditions eliminates the step of sterilizing the culture medium Thereby reducing the cost of LA production process. The study demonstrated that alternative proposed for LA production have potential for an industrial production process.

The process of LA separation and recovery of the fermentation broth was effective for removing contaminants and, at the end of the process, lactic acid was achieved an aqueous solution, around 416 g/l with 51% of yield. The LA recovery in this process can be applied on polymerization process of poly(lactic acid).

LA polycondensation process using different heating systems, conventional and microwave, resulted in polymers with different aspects and molecular weight. The developed polymerization process led to polymers in powder form (conventional process) and polymer in gel form (microwave process). Low molecular weight influences in the form of gel polymers. The energy required for polymerization using microwave is lower than using conventional heating process, furthermore a short time is required for reaction.

The polymers obtained in this work from monomers produced by fermentation and conventional heating polymerization presented physicochemical properties that demonstrate the possibility of the use of these materials in applications as encapsulation of bioactive compounds and tissue engineering.

Moreover, the polymers obtained by microwave heating polymerization, spite of its low weight and lower the Tg, exhibited similar infrared spectra, thus polymerization process is homogeneous and reproducible. These polymers form film high supported.

This work obtained promising results, particularly for the production of lactic acid. Polymerization is also a promising process because the polymers obtained, especially from monomer produced by fermentation, have physicochemical properties which enables their

application. There are prospects of studies to improve the process and obtaining polymers with enhanced properties.

FUTURE RESEARCH

These results were very promising because they present perspectives for studies sequence for obtaining a PLA by a new process. Thus, studies are performed to provide continuity are presented below:

- The scale up of the fermentation process to a pilot-scale plant;
- Improvement of the purification process in order to increase the yield and purity of lactic acid;
- Improvement of polymerization process and obtainment of high molecular weight PLA polymers;
- Studies of obtaining composite in order to improve physicochemical properties of the PLA polymers;
- Economic studies of the production costs for the products obtained.

REFERENCE

ABDEL-RAHMAN, M. A.; TASHIRO, Y.; SONOMOTO, K. Lactic acid production from lignocellulose-derived sugars using lactic acid bacteria: Overview and limits. **Journal of Biotechnology,** v. 156, n. 4, p. 286-301, 12/20/ 2011. ISSN 0168-1656.

_____. Recent advances in lactic acid production by microbial fermentation processes. **Biotechnology Advances,** v. 31, n. 6, p. 877-902, 11/1/ 2013. ISSN 0734-9750.

ABDEL-RAHMAN, M. A. et al. Fed-batch fermentation for enhanced lactic acid production from glucose/xylose mixture without carbon catabolite repression. **Journal of Bioscience and Bioengineering,** v. 119, n. 2, p. 153-158, 2// 2015. ISSN 1389-1723.

ABIKO, A.; YANO, S.-Y.; IGUCHI, M. Star-shaped poly(lactic acid) with carboxylic acid terminal groups via poly-condensation. v. 53, n. 18, p. 3842–3848, 17 August 2012 2012.

ACHMAD, F. et al. Synthesis of polylactic acid by direct polycondensation under vacuum without catalysts, solvents and initiators. **Chemical Engineering Journal,** v. 151, n. 1–3, p. 342-350, 8/15/ 2009. ISSN 1385-8947.

AESCHELMANN, F.; CARUS, M. **Bio-based Building Blocks and Polymers in the World Capacities, Production and Applications: Status Quo and Trends towards 2020** 2015.

AJIOKA, M. et al. The basic properties of poly(lactic acid) produced by the direct condensation polymerization of lactic acid. **Journal of environmental polymer degradation,** v. 3, n. 4, p. 225-234, 1995/10/01 1995. ISSN 1064-7546.

ALTAF, M.; NAVEENA, B. J.; REDDY, G. Use of inexpensive nitrogen sources and starch for l(+) lactic acid production in anaerobic submerged fermentation. **Bioresource Technology,** v. 98, n. 3, p. 498-503, 2// 2007. ISSN 0960-8524.

ARRIETA, M. P. et al. Characterization of PLA-limonene blends for food packaging applications. **Polymer Testing,** v. 32, n. 4, p. 760-768, 6// 2013. ISSN 0142-9418.

AURAS, R.; HARTE, B.; SELKE, S. An overview of polylactides as packaging materials. **Macromol Biosci,** v. 4, n. 9, p. 835-64, Sep 16 2004. ISSN 1616-5187 (Print)1616-5187.

AURAS, R. A. et al. Mechanical, Physical, and Barrier Properties of Poly(Lactide) Films. **Journal of Plastic Film and Sheeting,** v. 19, n. 2, p. 123-135, April 1, 2003 2003.

AVEROUS, L. Polylactic acid: Synthesis, properties and applications. In: BELGACEM, M. N. e GANDINI, A. (Ed.). **Monomers, Polymers and Composites from Renewable Resources**: Elsevier Science, 2008. cap. 21, p.433-450. ISBN 9780080560519.

BAO, L. et al. Gas permeation properties of poly(lactic acid) revisited. **Journal of Membrane Science,** v. 285, n. 1–2, p. 166-172, 11/15/ 2006. ISSN 0376-7388.

BAYAZIT, Ş. S.; İNCI, İ.; USLU, H. Adsorption of Lactic Acid from Model Fermentation Broth onto Activated Carbon and Amberlite IRA-67. **J. Chem. Eng. Data,** v. 56, n. 5, p. 1751-1754, February 25, 2011 2011.

BERGER, K.; GREGOROVA, A. Thermal stability of modified end-capped poly(lactic acid). **Journal of Applied Polymer Science,** v. 131, n. 22, p. n/a-n/a, 2014. ISSN 1097-4628.

BRITO, G. et al. Biopolímeros e polímeros verdes. **Revista Eletrônica de Materiais e Processos**, 2011.

BUDHAVARAM, N. K.; FAN, Z. Production of lactic acid from paper sludge using acid-tolerant, thermophilic Bacillus coagulan strains. **Bioresource Technology,** v. 100, n. 23, p. 5966–5972, December 2009 2009.

BUSTOS, G.; MOLDES, A. B.; CRUZ, J. M. Production of fermentable media from vine-trimming wastes and bioconversion into lactic acid by Lactobacillus pentosus. **Journal of the Science of Food and Agriculture,** v. 84, n. 15, p. 2105-2112, 2004. ISSN 1097-0010.

CAI, K. et al. Poly(d,l-lactic acid) surfaces modified by silk fibroin: effects on the culture of osteoblast in vitro. **Biomaterials,** v. 23, n. 4, p. 1153-1160, 2/15/ 2002. ISSN 0142-9612.

CAO, H. L.; WANG, P.; YUAN, W. B. Microwave-Assisted Synthesis of Poly(L-lactic acid) via Direct Melt Polycondensation Using Solid Super-Acids. **Macromolecular Chemistry and Physics,** v. 210, n. 23, p. 2058-2062, 2009. ISSN 1521-3935.

CARRASCO, F. et al. Processing of poly(lactic acid): Characterization of chemical structure, thermal stability and mechanical properties. **Polymer Degradation and Stability,** v. 95, n. 2, p. 116-125, 2// 2010. ISSN 0141-3910.

CARVALHO, W. T. D. et al. Drying kinetics of potato pulp waste. **Food Sci. Technol (Campinas),** v. 34, n. 1, p. 116-122, 03/2014 2014. ISSN 0101-2061.

CASTILLO MARTINEZ, F. A. et al. Lactic acid properties, applications and production: A review. **Trends in Food Science & Technology,** v. 30, n. 1, p. 70-83, 3// 2013. ISSN 0924-2244.

CHAUDHURI, J. B.; PHYLE, D. L. Emulsion liquid membrane extraction of organic acids—I. A theoretical model for lactic acid extraction with emulsion swelling. v. 47, n. 1, p. 41–48, January 1992 1992.

CHEAVEGATTI-GIANOTTO, A. et al. Sugarcane (Saccharum X officinarum): A Reference Study for the Regulation of Genetically Modified Cultivars in Brazil. **Tropical Plant Biology,** v. 4, n. 1, p. 62-89, 2011.

CHEN, G.-X. et al. Synthesis of high-molecular-weight poly(l-lactic acid) through the direct condensation polymerization of l-lactic acid in bulk state. **European Polymer Journal,** v. 42, n. 2, p. 468-472, 2// 2006. ISSN 0014-3057.

CONN, R. E. et al. Safety assessment of polylactide (PLA) for use as a food-contact polymer. **Food and Chemical Toxicology,** v. 33, n. 4, p. 273-283, 4// 1995. ISSN 0278-6915.

CONNOLLY, E.; ABRAHAMSSON, T.; BJÖRKSTÉN, B. Safety of D(-)-Lactic Acid Producing Bacteria in the Human I... : Journal of Pediatric Gastroenterology and Nutrition. **Journal of Pediatric Gastroenterology & Nutrition:,** v. 41, n. 4, p. 489-492, 2005.

CUI, F.; LI, Y.; WAN, C. Lactic acid production from corn stover using mixed cultures of Lactobacillus rhamnosus and Lactobacillus brevis. **Bioresour Technol,** v. 102, n. 2, p. 1831-6, Jan 2011. ISSN 0960-8524.

DATTA, R.; HENRY, M. Lactic acid: recent advances in products, processes and technologies — a review. **Journal of Chemical Technology & Biotechnology,** v. 81, n. 7, p. 1119-1129, 2006. ISSN 1097-4660.

DATTA, R. et al. Technological and economic potential of poly(lactic acid) and lactic acid derivatives. **FEMS Microbiology Reviews,** v. 16, n. 2–3, p. 221-231, 2// 1995. ISSN 0168-6445.

DEL NOBILE, M. A. et al. Active packaging by extrusion processing of recyclable and biodegradable polymers. **Journal of Food Engineering,** v. 93, n. 1, p. 1-6, 7// 2009. ISSN 0260-8774.

DENG, D. et al. Repair of Achilles tendon defect with autologous ASCs engineered tendon in a rabbit model. **Biomaterials,** v. 35, n. 31, p. 8801-8809, 10// 2014. ISSN 0142-9612.

DI LORENZO, M. L.; COCCA, M.; MALINCONICO, M. Crystal polymorphism of poly(l-lactic acid) and its influence on thermal properties. **Thermochimica Acta,** v. 522, n. 1–2, p. 110-117, 8/10/ 2011. ISSN 0040-6031.

DING, C. et al. Regeneration of a goat femoral head using a tissue-specific, biphasic scaffold fabricated with CAD/CAM technology. **Biomaterials,** v. 34, n. 28, p. 6706-6716, 9// 2013. ISSN 0142-9612.

DISTEL, K. A.; ZHU, G.; WANG, P. Biocatalysis using an organic-soluble enzyme for the preparation of poly(lactic acid) in organic solvents. **Bioresource Technology,** v. 96, n. 5, p. 617-623, 3// 2005. ISSN 0960-8524.

ENOMOTO, K.; AJIOKA, M.; YAMAGUCHI, A. **Polyhydroxycarboxylic acid and preparation process thereof** 1994.

EYAL, A. M.; BRESSLER, E. Industrial separation of carboxylic and amino acids by liquid membranes: Applicability, process considerations, and potential advantage. **Biotechnology and Bioengineering,** v. 41, n. 3, p. 287-295, 1993. ISSN 1097-0290.

FAMBRI, L.; MIGLIARESI, C. Crystallization and Thermal Properties. In: (Ed.). **Poly(Lactic Acid)**: John Wiley & Sons, Inc., 2010. p.113-124. ISBN 9780470649848.

FANG, J. M. et al. Development of biodegradable laminate films derived from naturally occurring carbohydrate polymers. **Carbohydrate Polymers,** v. 60, n. 1, p. 39-42, 4/7/ 2005. ISSN 0144-8617.

FORTUNATI, E. et al. Microstructure and nonisothermal cold crystallization of PLA composites based on silver nanoparticles and nanocrystalline cellulose. **Polymer Degradation and Stability,** v. 97, n. 10, p. 2027-2036, 10// 2012. ISSN 0141-3910.

FU, Y.-J. et al. Development of biodegradable co-poly(d,l-lactic/glycolic acid) microspheres for the controlled release of 5-FU by the spray drying method. **Colloids and Surfaces B: Biointerfaces,** v. 25, n. 4, p. 269-279, 8// 2002. ISSN 0927-7765.

GANAPATHY, H. S. et al. Ring-opening polymerization of l-lactide in supercritical carbon dioxide using PDMS based stabilizers. **European Polymer Journal,** v. 43, n. 1, p. 119-126, 1// 2007. ISSN 0014-3057.

GAO, C.; MA, C.; XU, P. Biotechnological routes based on lactic acid production from biomass. **Biotechnology Advances,** v. 29, n. 6, p. 930-939, 11// 2011. ISSN 0734-9750.

GARLOTTA, D. A Literature Review of Poly(Lactic Acid). **Journal of Polymers and the Environment,** v. 9, n. 2, p. 63-84, 2001/04/01 2001. ISSN 1566-2543.

GASPAR, P. et al. From physiology to systems metabolic engineering for the production of biochemicals by lactic acid bacteria. **Biotechnol Adv,** v. 31, n. 6, p. 764-88, Nov 2013. ISSN 0734-9750.

GHAFFAR, T. et al. Recent trends in lactic acid biotechnology: A brief review on production to purification. v. 7, n. 2, p. 222–229, April 2014 2014.

GIVRY, S.; PREVOT, V.; DUCHIRON, F. Lactic acid production from hemicellulosic hydrolyzate by cells of Lac. **World Journal of Microbiology and Biotechnology,** v. 24, n. 6, p. 745-752, 2007.

GRAUPNER, N.; HERRMANN, A. S.; MÜSSIG, J. Natural and man-made cellulose fibre-reinforced poly(lactic acid) (PLA) composites: An overview about mechanical characteristics and application areas. **Composites Part A: Applied Science and Manufacturing,** v. 40, n. 6–7, p. 810-821, 7// 2009. ISSN 1359-835X.

GRIGOROVA, R.; NORRIS, J. R. **Methods in Microbiology**. London: Academic Press Limited, 1990.

GROSS, R. A.; KUMAR, A.; KALRA, B. Polymer Synthesis by In Vitro Enzyme Catalysis. **Chemical Reviews,** v. 101, n. 7, p. 2097-2124, 2001/07/01 2001. ISSN 0009-2665.

GUO, Y. et al. Efficient production of lactic acid from sucrose and corncob hydrolysate by a newly isolated Rhizopus oryzae GY18. **Journal of Industrial Microbiology & Biotechnology,** v. 37, n. 11, p. 1137-1143, 2010/11/01 2010. ISSN 1367-5435.

GUPTA, A. P.; KUMAR, V. New emerging trends in synthetic biodegradable polymers – Polylactide: A critique. **European Polymer Journal,** v. 43, n. 10, p. 4053-4074, 10// 2007. ISSN 0014-3057.

HAMA, S. et al. Production of d-lactic acid from hardwood pulp by mechanical milling followed by simultaneous saccharification and fermentation using metabolically engineered Lactobacillus plantarum. **Bioresour Technol,** v. 187, p. 167-72, 2015. ISSN 0960-8524.

HENTON, D. E. et al. Polylactic acid technology. In: MOHANTY, A. K.;MISRA, M., *et al* (Ed.). **Natural Fibers, Biopolymers, and Biocomposites,** . Florida, The United States of America: Taylor & Francis, 2005. p.527-577.

HERRERA, N.; MATHEW, A. P.; OKSMAN, K. Plasticized polylactic acid/cellulose nanocomposites prepared using melt-extrusion and liquid feeding: Mechanical, thermal and optical properties. **Composites Science and Technology,** v. 106, n. 0, p. 149-155, 1/16/ 2015. ISSN 0266-3538.

HOFVENDAHL, K.; HAHN–HÄGERDAL, B. Factors affecting the fermentative lactic acid production from renewable resources 1. **Enzyme and microbial technology,** v. 26, n. 2, p. 87-107, 2000. ISSN 0141-0229.

HOOGENBOOM, R.; SCHUBERT, U. S. Microwave-Assisted Polymer Synthesis: Recent Developments in a Rapidly Expanding Field of Research. **Macromolecular Rapid Communications,** v. 28, n. 4, p. 368-386, 2007. ISSN 1521-3927.

HU, J. et al. Spectroscopic analysis on cold drawing-induced PLLA mesophase. **Polymer,** v. 53, n. 22, p. 4922-4926, 10/12/ 2012. ISSN 0032-3861.

HUTMACHER, D. W. Scaffolds in tissue engineering bone and cartilage. **Biomaterials,** v. 21, n. 24, p. 2529-2543, 12/15/ 2000. ISSN 0142-9612.

IDAGE, B. B. et al. Ring opening polymerization of dilactide using salen complex as catalyst. **Materials Science and Engineering: B,** v. 168, n. 1–3, p. 193-198, 4/15/ 2010. ISSN 0921-5107.

JAMSHIDIAN, M. et al. Poly-Lactic Acid: Production, Applications, Nanocomposites, and Release Studies. **Comprehensive Reviews in Food Science and Food Safety,** v. 9, n. 5, p. 552-571, 2010. ISSN 1541-4337.

JIMÉNEZ-BONILLA, P. et al. Optimization of Microwave-Assisted and Conventional Heating Comparative Synthesis of Poly(lactic acid) by Direct Melt Polycondensation from Agroindustrial Banana (Musa AAA Cavendish) and Pineapple (Ananas comosus) Fermented Wastes. **Journal of Polymers and the Environment,** v. 22, n. 3, p. 393-397, 2014/09/01 2014. ISSN 1566-2543.

JIN, T. et al. Antimicrobial activity of nisin incorporated in pectin and polylactic acid composite films against Listeria monocytogenes. **International Journal of Food Science & Technology,** v. 44, n. 2, p. 322-329, 2009. ISSN 1365-2621.

JING, S. et al. Microwave-irradiated ring-opening polymerization of D,L-lactide under atmosphere. **Journal of Applied Polymer Science,** v. 100, n. 3, p. 2244-2247, 2006. ISSN 1097-4628.

JING, S. et al. Rapid melt polycondensation of L-lactic acid under microwave irradiation. **Macromolecular Research,** v. 14, n. 6, p. 659-662, 2006/12/01 2006. ISSN 1598-5032.

JOGLEKAR, H. G. et al. Comparative assessment of downstream processing options for lactic acid. v. 52, n. 1, p. 1–17, November 2006 2006.

JOHN, R. P.; NAMPOOTHIRI, K. M.; PANDEY, A. Solid-state fermentation for l-lactic acid production from agro wastes using Lactobacillus delbrueckii. v. 41, n. 4, p. 759–763, April 2006 2006.

_____. Fermentative production of lactic acid from biomass: an overview on pr. **Applied Microbiology and Biotechnology,** v. 74, n. 3, p. 524-534, 2007.

KAPPE, C. O. Controlled Microwave Heating in Modern Organic Synthesis. **Angewandte Chemie International Edition,** v. 43, n. 46, p. 6250-6284, 2004. ISSN 1521-3773.

KARP, S. G. et al. Application of the biorefinery concept to produce l-lactic acid from the soybean vinasse at laboratory and pilot scale. **Bioresource Technology,** v. 102, n. 2, p. 1765-1772, 1// 2011. ISSN 0960-8524.

KATIYAR, V.; NANAVATI, H. High molecular weight poly (L-lactic acid) clay nanocomposites via solid-state polymerization. **Polymer Composites,** v. 32, n. 3, p. 497-509, 2011. ISSN 1548-0569.

KÉKI, S. et al. Fast Microwave-Mediated Bulk Polycondensation of d,l-Lactic Acid. **Macromolecular Rapid Communications,** v. 22, n. 13, p. 1063-1065, 2001. ISSN 1521-3927.

KHUNNONKWAO, P. et al. Purification of l-(+)-lactic acid from pre-treated fermentation broth using vapor permeation-assisted esterification. **Process Biochemistry,** v. 47, n. 12, p. 1948–1956, December 2012 2012.

KIRAN, K.; DIVAKAR, S. Lipase-catalysed polymerization of lactic acid and its film forming properties. **World Journal of Microbiology and Biotechnology,** v. 19, n. 8, p. 859-865, 2003/11/01 2003. ISSN 0959-3993.

KIRBY, B. P. et al. Comparative evaluation of the degree of pegylation of poly(lactic-co-glycolic acid) nanoparticles in enhancing central nervous system delivery of loperamide. **Journal of Pharmacy and Pharmacology,** v. 65, n. 10, p. 1473-1481, 2013. ISSN 2042-7158.

KOBAYASHI, S.; MAKINO, A. Enzymatic Polymer Synthesis: An Opportunity for Green Polymer Chemistry. **Chemical Reviews,** v. 109, n. 11, p. 5288-5353, 2009/11/11 2009. ISSN 0009-2665.

KOBAYASHI, S.; UYAMA, H.; KIMURA, S. Enzymatic Polymerization. **Chemical Reviews,** v. 101, n. 12, p. 3793-3818, 2001/12/01 2001. ISSN 0009-2665.

KODAMA, Y. et al. Investigation of irradiated biodegradable blends by FTIR and wide-angle X-ray diffraction. **Nukleonika,** v. 54, p. 107-113, 2009. ISSN 0029-5922.

KRIKORIAN, V.; POCHAN, D. J. Crystallization behavior of poly (L-lactic acid) nanocomposites: nucleation and growth probed by infrared spectroscopy. **Macromolecules,** v. 38, n. 15, p. 6520-6527, 2005. ISSN 0024-9297.

KRZYŻANIAK, A. et al. Novel extractants for the recovery of fermentation derived lactic acid. **Separation and Purification Technology,** v. 111, p. 82–89, 25 June 2013 2013.

KUBANTSEVA, N.; HARTEL, R. W. Solubility of calcium lactate in aqueous solution. **Food Reviews International,** v. 18, n. 2, p. 135-149, 2011-11-02 2002.

KUMAR, M. et al. Effect of glycidyl methacrylate (GMA) on the thermal, mechanical and morphological property of biodegradable PLA/PBAT blend and its nanocomposites. **Bioresource Technology,** v. 101, n. 21, p. 8406-8415, 11// 2010. ISSN 0960-8524.

KYLÄ-NIKKILÄ, K. et al. Metabolic Engineering of Lactobacillus helveticus CNRZ32 for Production of Pure l-(+)-Lactic Acid. In: (Ed.). **Appl Environ Microbiol**, v.66, 2000. p.3835-41. ISBN 0099-2240 (Print)1098-5336 (Electronic).

LAHTINEN, S. et al. **Lactic Acid Bacteria: Microbiological and Functional Aspects**. 4. Boca Raton: CRC Press, 2011. 798 ISBN 1439836787, 9781439836781.

LANTANO, C. et al. Natamycin based sol–gel antimicrobial coatings on polylactic acid films for food packaging. **Food Chemistry,** v. 165, n. 0, p. 342-347, 12/15/ 2014. ISSN 0308-8146.

LASPRILLA, A. J. R.; MARTINEZ, G. A. R.; HOSS, B. Synthesis and Characterization of Poly (lactic acid) for Use in Biomedical Field. **Chem. Eng,** v. 24, p. 985-990, 2011.

LASPRILLA, A. J. R. et al. Poly-lactic acid synthesis for application in biomedical devices — A review. **Biotechnology Advances,** v. 30, n. 1, p. 321-328, 1// 2012. ISSN 0734-9750.

LE MOIGNE, N. et al. Study of the interface in natural fibres reinforced poly(lactic acid) biocomposites modified by optimized organosilane treatments. **Industrial Crops and Products,** v. 52, n. 0, p. 481-494, 1// 2014. ISSN 0926-6690.

LEHERMEIER, H. J.; DORGAN, J. R.; WAY, J. D. Gas permeation properties of poly(lactic acid). **Journal of Membrane Science,** v. 190, n. 2, p. 243-251, 9/15/ 2001. ISSN 0376-7388.

LEJA, K.; LEWANDOWICZ, G. Polymer biodegradation and biodegradable polymers—a review. **Polish Journal of Environmental Studies,** v. 19, n. 2, p. 255-266, 2010. ISSN 1230-1485.

LI, H. et al. An electrokinetic bioreactor: using direct electric current for enhanced lactic acid fermentation and product recovery. **Tetrahedron,** v. 60, n. 3, p. 655–661, 12 January 2004 2004.

LIM, L. T.; AURAS, R.; RUBINO, M. Processing technologies for poly(lactic acid). **Progress in Polymer Science,** v. 33, n. 8, p. 820-852, 8// 2008. ISSN 0079-6700.

LIU, H. et al. Interaction of Microstructure and Interfacial Adhesion on Impact Performance of Polylactide (PLA) Ternary Blends. **Macromolecules,** v. 44, n. 6, p. 1513-1522, 2011/03/22 2011. ISSN 0024-9297.

LÜ, J.-M. et al. Current advances in research and clinical applications of PLGA-based nanotechnology. **Expert Review of Molecular Diagnostics,** v. 9, n. 4, p. 325-341, 2009.

LUCAS, E. F.; SOARES, B. G.; MONTEIRO, E. **Caracterização de Polímeros: Determinação de Peso Molecular e Análise Térmica**. Rio do Janeiro: E-papers, 2001. 366.

LUNELLI, B. H. et al. Production of Lactic Acid from Sucrose: Strain Selection, Fermentation. **Applied Biochemistry and Biotechnology,** v. 161, n. 1, p. 227-237, 2009.

MA, K. et al. Open fermentative production of l-lactic acid with high optical purity by thermophilic Bacillus coagulans using excess sludge as nutrient. v. 151, p. 28–35, January 2014 2014.

MA, Z. et al. Protein immobilization on the surface of poly-L-lactic acid films for improvement of cellular interactions. **European Polymer Journal,** v. 38, n. 11, p. 2279-2284, 11// 2002. ISSN 0014-3057.

MADHAVAN NAMPOOTHIRI, K.; NAIR, N. R.; JOHN, R. P. An overview of the recent developments in polylactide (PLA) research. **Bioresource Technology,** v. 101, n. 22, p. 8493-8501, 11// 2010. ISSN 0960-8524.

MALAFAYA, P. B.; SILVA, G. A.; REIS, R. L. Natural–origin polymers as carriers and scaffolds for biomolecules and cell delivery in tissue engineering applications. **Advanced Drug Delivery Reviews,** v. 59, n. 4–5, p. 207-233, 5/30/ 2007. ISSN 0169-409X.

MARQUES, D. A. S. et al. Poly(lactic acid) Synthesis in Solution Polymerization. **Macromolecular Symposia,** v. 296, n. 1, p. 63-71, 2010. ISSN 1521-3900.

MARTÁK, J.; SCHLOSSER, Š.; VLČKOVÁ, S. Pertraction of lactic acid through supported liquid membranes containing phosphonium ionic liquid ☆. **Journal of Membrane Science,** v. 318, n. Issues 1–2, p. 298–310, 20 June 2008 2008.

MARTÍNEZ-ABAD, A.; LAGARÓN, J. M.; OCIO, M. J. Antimicrobial beeswax coated polylactide films with silver control release capacity. **International Journal of Food Microbiology,** v. 174, n. 0, p. 39-46, 3/17/ 2014. ISSN 0168-1605.

MARTINO, V. P. et al. Structure and properties of clay nano-biocomposites based on poly(lactic acid) plasticized with polyadipates. **Polymers for Advanced Technologies,** v. 22, n. 12, p. 2206-2213, 2011. ISSN 1099-1581.

MASCHERONI, E. et al. Diffusivity of propolis compounds in Polylactic acid polymer for the development of anti-microbial packaging films. **Journal of Food Engineering,** v. 98, n. 3, p. 294-301, 2010. ISSN 0260-8774.

MAZUMDAR, S. et al. Efficient synthesis of L-lactic acid from glycerol by metabolically engineered Escherichia coli. **Microb Cell Fact,** v. 12, p. 7, 2013. ISSN 1475-2859.

MAZZOLI, R. et al. Towards lactic acid bacteria-based biorefineries. **Biotechnology Advances,** v. 32, n. 7, p. 1216-1236, 11/15/ 2014. ISSN 0734-9750.

MENG, Y. et al. Efficient production of l-lactic acid with high optical purity by alkaliphilic Bacillus sp. WL-S20. **Bioresource Technology,** v. 116, n. 0, p. 334-339, 7// 2012. ISSN 0960-8524.

MIDDLETON, J. C.; TIPTON, A. J. Synthetic biodegradable polymers as orthopedic devices. **Biomaterials,** v. 21, n. 23, p. 2335-2346, 12/1/ 2000. ISSN 0142-9612.

MIN, D.-J. et al. Effect of operating parameters on precipitation for recovery of lactic. **Korean Journal of Chemical Engineering,** v. 28, n. 10, p. 1969-1974, 2011.

MOON, S.-K.; WEE, Y.-J.; CHOI, G.-W. A novel lactic acid bacterium for the production of high purity l-lactic acid, Lactobacillus paracasei subsp. paracasei CHB2121. **Journal of Bioscience and Bioengineering,** v. 114, n. 2, p. 155-159, 8// 2012. ISSN 1389-1723.

MOON, S.-K. W., YOUNG-JUNG CHOI, GI-WOOK. A novel lactic acid bacterium for the production of high purity l-lactic acid, Lactobacillus paracasei subsp. paracasei CHB2121. v. 114, n. 2, p. 155–159, August 2012 2012.

MOTTA, A. C.; DUEK, E. A. R. Síntese, caracterização e degradação " in vitro" do Poli(L-ácido láctico). **Polímeros,** v. 16, p. 26-32, 2006. ISSN 0104-1428.

NAGAHATA, R. et al. Microwave-Assisted Single-Step Synthesis of Poly(lactic acid) by Direct Polycondensation of Lactic Acid. **Macromolecular Rapid Communications,** v. 28, n. 4, p. 437-442, 2007. ISSN 1521-3927.

NAKAMURA, T. et al. Large-Scale Polycondensation of Lactic Acid Using Microwave Batch Reactors. **Organic Process Research & Development,** v. 14, n. 4, p. 781-786, 2010/07/16 2010. ISSN 1083-6160.

NANAVATI, H.; KATIYAR, V. **Method for producing lactic acid polymers of high crystallinity and molecular weight** 2010.

NANCIB, A.; NANCIB, N.; BOUDRANT, J. Production of lactic acid from date juice extract with free cells of s. **World Journal of Microbiology and Biotechnology,** v. 25, n. 8, p. 1423-1429, 2009.

NANCIB, A. et al. Joint effect of nitrogen sources and B vitamin supplementation of date juice on lactic acid production by Lactobacillus casei subsp. rhamnosus. v. 96, n. 1, p. 63–67, January 2005 2005.

NARAYANAN, N.; ROYCHOUDHURY, P. K.; SRIVASTAVA, A. L (+) lactic acid fermentation and its product polymerization. **Electronic Journal of Biotechnology,** v. 7, p. 167-178, 2004. ISSN 0717-3458.

NOMURA, Y.; YAMAMOTO, K.; ISHIZAKI, A. Factors affecting lactic acid production rate in the built-in electrodialysis fermentation, an approach to high speed batch culture. **Journal of Fermentation and Bioengineering,** v. 71, n. 6, p. 450-452, 1991.

OBUCHI, S.; OGAWA, S. Packaging and other commercial applications. In: AURAS, R.;LIM, L. T., *et al* (Ed.). **Poly(lactic acid): Synthesis, Structures, Properties, Processing, and Applications**. New Jersey: John Wiley & Sons, Inc, 2010. p.528. ISBN 978-0-470-29366-9.

OH, S. H. et al. Fabrication and characterization of hydrophilic poly(lactic-co-glycolic acid)/poly(vinyl alcohol) blend cell scaffolds by melt-molding particulate-leaching method. **Biomaterials,** v. 24, n. 22, p. 4011-4021, 10// 2003. ISSN 0142-9612.

OKANO, K. et al. Homo-d-Lactic Acid Fermentation from Arabinose by Redirection of the Phosphoketolase Pathway to the Pentose Phosphate Pathway in l-Lactate Dehydrogenase Gene-Deficient Lactobacillus plantarum▽. **Appl Environ Microbiol,** v. 75, n. 15, p. 5175-5178, 2009. ISSN 0099-2240 (Print)1098-5336 (Electronic).

OLIVEIRA, A. C. G. D. et al. Effects of heat treatment and gamma radiation on the characteristics of pure sugarcane juice and mixed with fruit juices. **Food Sci. Technol (Campinas),** v. 27, n. 4, p. 863-873, 12/2007 2007. ISSN 0101-2061.

OROZCO, F. G. et al. Lactic Acid Yield Using Different Bacterial Strains, Its Purification, and Polymerization through Ring-Opening Reactions. **International Journal of Polymer Science,** v. 2014, p. 7, 2014.

OROZCO, V. H.; VARGAS, A. F.; LÓPEZ, B. L. Study of the Polymerization Kinetic of Lactic Acid. **Macromolecular Symposia,** v. 258, n. 1, p. 45-52, 2007. ISSN 1521-3900.

OUYANG, J. et al. Open fermentative production of l-lactic acid by Bacillus sp. strain NL01 using lignocellulosic hydrolyzates as low-cost raw material. **Bioresource Technology,** v. 135, p. 475–480, May 2013 2013.

ÖZGE ERDOHAN, Z.; ÇAM, B.; TURHAN, K. N. Characterization of antimicrobial polylactic acid based films. **Journal of Food Engineering,** v. 119, n. 2, p. 308-315, 11// 2013. ISSN 0260-8774.

PANDEY, A.; ASWATH, P. B. Microwave Synthesis of Poly(L-lactic acid). **Journal of Biomaterials Science, Polymer Edition,** v. 20, n. 1, p. 33-48, 2009/01/01 2009. ISSN 0920-5063.

PEREGO, G.; CELLA, G. D.; BASTIOLI, C. Effect of molecular weight and crystallinity on poly(lactic acid) mechanical properties. **Journal of Applied Polymer Science,** v. 59, n. 1, p. 37-43, 1996. ISSN 1097-4628.

PEREIRA, R. B.; MORALES, A. R. Estudo do comportamento térmico e mecânico do PLA modificado com aditivo nucleante e modificador de impacto. **Polímeros,** v. 24, p. 198-202, 2014. ISSN 0104-1428.

PETINAKIS, E. et al. Biodegradation and thermal decomposition of poly(lactic acid)-based materials reinforced by hydrophilic fillers. **Polymer Degradation and Stability,** v. 95, n. 9, p. 1704-1707, 9// 2010. ISSN 0141-3910.

PIVSA-ART, S. et al. Synthesis of Poly(D-Lactic Acid) Using a 2-Steps Direct Polycondensation Process. **Energy Procedia,** v. 34, n. 0, p. 604-609, // 2013. ISSN 1876-6102.

PREMRAJ, R.; DOBLE, M. Biodegradation of polymers. **Indian Journal of Biotechnology,** v. 4, n. 2, p. 186-193, 2005.

QIN, J. et al. Production of l-lactic acid by a thermophilic Bacillus mutant using sodium hydroxide as neutralizing agent. **Bioresource Technology,** v. 101, n. 19, p. 7570-7576, 10// 2010. ISSN 0960-8524.

RAFAT, M. et al. PEG–PLA microparticles for encapsulation and delivery of Tat-EGFP to retinal cells. **Biomaterials,** v. 31, n. 12, p. 3414-3421, 4// 2010. ISSN 0142-9612.

REDDY, G. et al. Amylolytic bacterial lactic acid fermentation — A review. **Biotechnology Advances,** v. 26, n. 1, p. 22-34, 1// 2008. ISSN 0734-9750.

RESEARCH, G. V. Lactic Acid And Poly Lactic Acid (PLA) Market Analysis By Application (Packaging, Agriculture, Transport, Electronics, Textiles) And Segment Forecasts To 2020. San Francisco, 2015. Disponível em: < https://www.grandviewresearch.com/press-release/global-lactic-acid-and-poly-lactic-acid-market >.

SANTOS, F. A. D.; TAVARES, M. I. B. Preparo e caracterização de filmes obtidos a partir de poli(ácido lático) e celulose microcristalina. **Polímeros,** v. 23, p. 229-235, 2013. ISSN 0104-1428.

SCHWACH, G. et al. Ring opening polymerization of D,L-lactide in the presence of zinc metal and zinc lactate. **Polymer International,** v. 46, n. 3, p. 177-182, 1998. ISSN 1097-0126.

SHINNO, K. et al. Solid-State Postpolymerization of l-Lactide Promoted by Crystallization of Product Polymer: An Effective Method for Reduction of Remaining Monomer. **Macromolecules,** v. 30, n. 21, p. 6438-6444, 1997/10/01 1997. ISSN 0024-9297.

SHYAMROY, S.; GARNAIK, B.; SIVARAM, S. Structure of poly(L-lactic acid)s prepared by the dehydropolycondensation of L-lactic acid with organotin catalysts. **Journal of Polymer Science Part A: Polymer Chemistry,** v. 43, n. 10, p. 2164-2177, 2005. ISSN 1099-0518.

SINGH, S. K.; AHMED, S. U.; PANDEY, A. Metabolic engineering approaches for lactic acid production. v. 41, n. 5, p. 991–1000, May 2006 2006.

SINGLA, P. et al. Ring-Opening Polymerization of Lactide Using Microwave and Conventional Heating. **Procedia Chemistry,** v. 4, n. 0, p. 179-185, // 2012. ISSN 1876-6196.

SIRACUSA, V. et al. Poly(lactic acid)-modified films for food packaging application: Physical, mechanical, and barrier behavior. **Journal of Applied Polymer Science,** v. 125, n. S2, p. E390-E401, 2012. ISSN 1097-4628.

SÖDERGÄRD, A.; STOLT, M. Industrial Production Of High Molecular Weight Poly(Lactic Acid). In: AURAS, R.;LIM, L. T., *et al* (Ed.). **Poly(lactic acid): Synthesis, Structures, Properties, Processing, and Applications.** New Jersey, United States of America: John Wiley & Sons, Inc., 2010. p.528.

SÖDERGÅRD, A.; STOLT, M. Properties of lactic acid based polymers and their correlation with composition. **Progress in Polymer Science,** v. 27, n. 6, p. 1123–1163, July 2002 2002.

SOSNIK, A.; GOTELLI, G.; ABRAHAM, G. A. Microwave-assisted polymer synthesis (MAPS) as a tool in biomaterials science: How new and how powerful. **Progress in Polymer Science,** v. 36, n. 8, p. 1050-1078, 8// 2011. ISSN 0079-6700.

STOLT, M.; SÖDERGÅRD, A. Use of Monocarboxylic Iron Derivatives in the Ring-Opening Polymerization of l-Lactide. **Macromolecules,** v. 32, n. 20, p. 6412-6417, 1999/10/01 1999. ISSN 0024-9297.

SUJARITJUN, W. et al. Mechanical Property of Surface Modified Natural Fiber Reinforced PLA Biocomposites. **Energy Procedia,** v. 34, n. 0, p. 664-672, // 2013. ISSN 1876-6102.

SUZUKI, S.; IKADA, Y. Medical Applications in In: AURAS, R.;LIM, L. T., *et al* (Ed.). **Poly(lactic acid): Synthesis, Structures, Properties, Processing, and Applications**. New Jersey, United States of America: John Wiley & Sons, Inc., , 2010. p.528.

TABI, T. et al. Crystalline structure of annealed polylactic acid and its relation to processing. **Express Polym Lett,** v. 4, n. 10, p. 659-668, 2010.

TALUKDER, M. M. R.; DAS, P.; WU, J. C. Microalgae (Nannochloropsis salina) biomass to lactic acid and lipid. **Biochemical Engineering Journal,** v. 68, p. 109–113, 15 October 2012 2012.

TANAKA, K. et al. Two different pathways for D-xylose metabolism and the effect of xylos. **Applied Microbiology and Biotechnology,** v. 60, n. 1, p. 160-167, 2002.

TANIGUCHI, M. et al. Production of L-lactic acid from a mixture of xylose and glucose by co-cultivation of lactic acid bacteria. **Appl Microbiol Biotechnol,** v. 66, n. 2, p. 160-5, Dec 2004. ISSN 0175-7598 (Print)0175-7598.

TASHIRO, Y. et al. Continuous D-lactic acid production by a novel thermotolerant Lactobacillus delbrueckii subsp. lactis QU 41. **Appl Microbiol Biotechnol,** v. 89, n. 6, p. 1741-50, Mar 2011. ISSN 0175-7598.

THANG, V. H.; NOVALIN, S. Green biorefinery: separation of lactic acid from grass silage juice by chromatography using neutral polymeric resin. **Bioresour Technol,** v. 99, n. 10, p. 4368-79, Jul 2008. ISSN 0960-8524 (Print)0960-8524.

TIAN, K. et al. High-efficiency conversion of glycerol to D-lactic acid with metabolically engineered Escherichia coli. **African Journal of Biotechnology,** v. 11, n. 21, p. 4860-4867, 2012.

UHRICH, K. E. et al. Polymeric systems for controlled drug release. **Chemical reviews,** v. 99, n. 11, p. 3181-3198, 1999. ISSN 0009-2665.

VARMA, I. K. et al. Enzyme catalyzed synthesis of polyesters. **Progress in Polymer Science,** v. 30, n. 10, p. 949-981, 10// 2005. ISSN 0079-6700.

VENDRUSCOLO, J. L. S.; ZORZELLA, C. A. **Processamento de batata (Solanum tuberosum L.) : fritura - Portal Embrapa**: Embrapa Clima Temperado**:** 15 p. 2002.

VIJAYAKUMAR, J.; ARAVINDAN, R.; VIRUTHAGIRI, T. Recent Trends in the Production, Purification and Application of Lactic Acid. **Chemical and Biochemical Engineering Quarterly,** v. 22, n. 2, p. 245-264, 2008. ISSN 0352-9568.

VOUYIOUKA, S. et al. Solid state polymerization of poly(lactic acid): Some fundamental parameters. **Polymer Degradation and Stability,** v. 98, n. 12, p. 2473-2481, 12// 2013. ISSN 0141-3910.

VOUYIOUKA, S. N.; KARAKATSANI, E. K.; PAPASPYRIDES, C. D. Solid state polymerization. **Progress in Polymer Science,** v. 30, n. 1, p. 10-37, 1// 2005. ISSN 0079-6700.

WANG, L. et al. Highly efficient production of d-lactate by Sporolactobacillus sp. CASD with simultaneous enzymatic hydrolysis of peanut meal. **Applied Microbiology and Biotechnology,** v. 89, n. 4, p. 1009-1017, 2011/02/01 2011. ISSN 0175-7598.

WANG, L. et al. Efficient production of l-lactic acid from cassava powder by Lactobacillus rhamnosus. **Bioresource Technology,** v. 101, n. 20, p. 7895-7901, 10// 2010. ISSN 0960-8524.

WANG, Y. et al. Homofermentative production of D-lactic acid from sucrose by a metabolically engineered Escherichia coli. **Biotechnol Lett,** v. 34, n. 11, p. 2069-75, Nov 2012. ISSN 0141-5492.

WASEWAR, K. L. Separation of lactic acid : Recent advances. **Chemical and biochemical engineering quarterly,** v. 19, n. 2, p. 14, 2005.

WATANABE, S. et al. New microwave-assisted rapid synthesis of polyamides from nylon salts. **Die Makromolekulare Chemie, Rapid Communications,** v. 14, n. 8, p. 481-484, 1993. ISSN 0173-2803.

WEE, Y.-J.; KIM, J.-N.; RYU, H.-W. Biotechnological production of lactic acid and its recent applications. **Food Technology and Biotechnology,** v. 44, n. 2, p. 163-172, 2006. ISSN 1330-9862.

WOICIECHOWSKI, A. L. et al. Acid and enzymatic hydrolysis to recover reducing sugars from cassava bagasse: an economic study. **Brazilian Archives of Biology and Technology,** v. 45, n. 3, p. 393-400, 09/2002 2002. ISSN 1516-8913.

YADAV, A. K.; CHAUDHARI, A. B.; KOTHARI, R. M. Bioconversion of renewable resources into lactic acid: an industrial view. **Critical Reviews in Biotechnology,** v. 31, n. 1, p. 1-19, 2011.

YAMADA, S. et al. Microwave-assisted solution polycondensation of l-lactic acid using a Dean-Stark apparatus for a non-thermal microwave polymerization effect induced by the electric field. **Polymer Chemistry,** v. 5, n. 18, p. 5283-5288, 2014. ISSN 1759-9954.

YASUNIWA, M. et al. Thermal analysis of the double-melting behavior of poly(L-lactic acid). **Journal of Polymer Science Part B: Polymer Physics,** v. 42, n. 1, p. 25-32, 2004. ISSN 1099-0488.

YOSHIDA, S. et al. Homo-D-lactic acid production from mixed sugars using xylose-assimilating operon-integrated Lactobacillus plantarum. **Appl Microbiol Biotechnol,** v. 92, n. 1, p. 67-76, Oct 2011. ISSN 0175-7598.

ZAFAR, M. et al. Biocomposites based on poly (lactic acid)/willow-fiber and their injection moulded microcellular foams. **Express Polymer Letters,** v. 10, n. 2, 2016. ISSN 1788-618X.

ZHAI, W. et al. A study of the crystallization, melting, and foaming behaviors of polylactic acid in compressed CO(2). **Int J Mol Sci,** v. 10, n. 12, p. 5381-97, Dec 2009. ISSN 1422-0067.

ZHANG, J. et al. Crystal Modifications and Thermal Behavior of Poly(l-lactic acid) Revealed by Infrared Spectroscopy. **Macromolecules,** v. 38, n. 19, p. 8012-8021, 2005/09/01 2005. ISSN 0024-9297.

ZHANG, L. et al. Simultaneous saccharification and fermentation of xylo-oligosaccharides manufacturing waste residue for l-lactic acid production by Rhizopus oryzae. **Biochemical Engineering Journal,** v. 94, p. 92–99, 15 February 2015 2015.

ZHANG, Y.; CHAN, H. F.; LEONG, K. W. Advanced materials and processing for drug delivery: The past and the future. **Advanced Drug Delivery Reviews,** v. 65, n. 1, p. 104-120, 1// 2013. ISSN 0169-409X.

ZHAO, J. et al. Homofermentative production of optically pure L-lactic acid from xylose by genetically engineered Escherichia coli B. **Microb Cell Fact,** v. 12, p. 57, 2013. ISSN 1475-2859.

ZHU, Y.; LEE, Y. Y.; ELANDER, R. T. Conversion of aqueous ammonia-treated corn stover to lactic acid by simultaneous saccharification and cofermentation. **Appl Biochem Biotechnol,** v. 137-140, n. 1-12, p. 721-38, Apr 2007. ISSN 0273-2289.